SEROLOGICAL MARKERS HBsAg AND HBeAg IN CHRONIC HEPATITIS B CARRIERS AND THEIR CORRELATION WITH VIRAL LOAD BY POLYMERASE CHAIN REACTION ASSAY

By

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MD

IN

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LIST OF ABBREVIATIONS

ABBREVIATION	EXPANSION
DNA	Deoxyribonucleic acid
HIV	Human Immunodeficiency Virus
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
CHB	Chronic Hepatitis B
HBsAg	Hepatitis B surface Antigen
HBeAg	Hepatitis B e Antigen
PCR	Polymerase Chain Reaction
ALT	Alanine aminotransferase
Anti-HBc	Anti- Hepatitis B core antibody
WHO	World Health Organization
HBx	Hepatitis B x
RNA	Ribonucleic Acid
PEG-IFN	Pegylated Interferon
ccc	Covalently closed circular
mRNA	Messenger RNA
S,M,L FORMS	Small, Medium, Large
PreC/C	Pre-core/core region
CD	Cluster of Differentiation
IgM,G	Immunoglobulin M,G
RT-PCR	Real Time Polymerase Chain Reaction

HBIG	Hepatitis B Immunoglobulin
AFP	Alpha feto protein
IU/ml	International Units/ml

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INTRODUCTION

Hepatitis B is the most widespread and the most important type of viral hepatitis[1]. According to WHO estimate, there are now 400,000,000 individuals world-wide who are chronically infected with HBV,25% of whom may die of chronic liver disease or hepatocellular carcinoma[2].In India, the carrier rate has been found to be around 4-7% with an estimated 45 million individuals who are infected with this virus.[3]

The hepatitis B virus resides in human carrier pool and has sporadic infections, though outbreaks in mental hospitals, orphanages and hospitals are known to occur.[1]

Hepatitis B virus is present in saliva, semen, blood, vaginal secretions, and to a smaller extent in perspiration, breast milk, tears and urine of infected persons. The virus is transmitted horizontally through sexual contact, contact with body fluids, blood and blood products, needle pricks and vertically from mother to fetus. Hepatitis B virus infection leads to a wide range of liver diseases extending from acute to chronic hepatitis, cirrhosis and hepatocellular carcinoma.[3]

A carrier is a person in whom HBsAg is detectable in blood for more than 6 months.[1]

Infection at birth usually manifests with clinically silent acute infection, but a 90% chance of chronic infection, while infection in young adulthood in immune competent person is most commonly associated with clinically apparent acute hepatitis, but the risk of chronicity is much lesser, approximately 1%.[4]

Carriers are of two types: 1) Super carriers and 2) Simple carriers.[1]

Serological markers are indispensible in the diagnosis of Hepatitis B virus infection, but HBsAg does not provide information about active virus replication. Viral

load in an individual gives exact assessment of disease, its progression, efficacy of treatment, emergence of resistant forms and true carrier status of the individual.[4]

HBeAg is a soluble non-particulate nucleocapsid protein. It is present in the circulation and the presence of HBeAg in the blood provides a convenient and easily detectable marker of HBV replication and high infectivity of the carrier. In HBeAg reactive chronic hepatitis B, 2 groups have been established.

a) **Replicative:** Virus load ranging from 10^3 -10^4/ml up to 10^9 /ml.[1]

b) **Non- Replicative:** Viral DNA is below 10^3/ml.[1]

However, due to mutation in pre core region, there is inability to synthesize HBeAg, whereas viral load continues to increase in the patient. The most common irony in the case of chronic hepatitis B patients is the differentiation between HBeAg negative chronic hepatitis cases from inactive carriers as they share their serological profile.[4]

The determination of HBV in plasma and serum however is superior to HBeAg testing as it reflects the replicative activity of HBV more accurately. The level of HBV replication is the most important risk factor for ultimate development of cirrhosis and hepatocellular carcinoma in both HBeAg reactive and HBeAg nonreactive patients.[4]

As the carrier state is asymptomatic, it is diagnosed incidentally during pre-operative screening, ante-natal check- up, blood-donation camps and when, a family member is found positive and all the others are screened. These people may be responsible for the spread of infection in the community.

Also, this phase is not static and in the presence of transient immune status fluctuations, may become clinically overt and severe. Hence these patients have to be monitored regularly.

In view of these facts, this study has been planned to assess the serological markers, HBsAg and HBeAg in the chronic carriers of hepatitis B and compare them with viral load as determined by Polymerase chain reaction assay.

AIMS AND OBJECTIVES

1) To detect serologically the presence of HBsAg and HBeAg in the blood of chronic hepatitis B patients
2) To assess the viral load by polymerase chain reaction assay.
3) To correlate the presence of HBsAg and HBeAg with viral DNA load.

REVIEW OF LITERATURE

HISTORY

The spread of jaundice by infectious agents was recognized by Hippocrates as early as 4000 B.C.

The possibility of accidental transmission of hepatitis by blood was recognized in 1925 in Sweden among diabetic patients who attended a laboratory for blood tests.[5]

Hepatitis B Virus was recognized as a causative agent for serum hepatitis around mid-60`s. These studies were performed by Krugman and colleagues at the Willow brook School for mentally handicapped children. Dr. Baruch Blumberg reported the discovery of a human antigen in Australian Aborigines termed Australia antigen for which he received the Nobel Prize for Physiology and Medicine. Blumberg and Prince identified the connection between Australia antigen and HBV.

Dane, in 1970 visualized infectious virion or Dane particle and Almeida saw nucleocapsid core by electron microscope. In the early 1970 s, the virus genome was characterized, and a study of serological profile of chronic HBV infection was done by Robinson`s group at Stanford. The connection between the virus and HCC was established at the same time.

Galbraith in 1975 reported, that in three patients with malignant diseases, upon withdrawal of cytotoxic drugs, fulminant hepatitis followed, resulting in death. Their serum had tested positive for HBsAg at least 6 months before the development of acute Hepatitis B[6].

In 1978, three scientists, Pierre Tiollais (Paris), William Rutler (San Francisco) and Kenneth Murray (Edinburgh) almost simultaneously reported cloning and sequencing of HBV DNA[7].

In 1980`s, development of genetic engineering took place and the genome and its replication strategy was also understood.

Friedrich Deinhardt conducted animal experiments and proved that Dane particle is the HBV, according to Koch`s postulate in 1982.

The role of PCR in the field is of paramount importance.

In 1987, Carmen and colleagues described the molecular basis of HBe antigen negative viremia and the first vaccine escape mutant (arginine 145) in vaccinated children born to HBsAg positive mothers was also described by them.

Szmuness and his team studied in detail the plasma derived vaccine in chimpanzees.

The production of the recombinant vaccine soon followed. Interferons were used in the treatment of chronic hepatitis B for the first time during that period. [8]

HEPATITIS B EPIDEMIOLOGY

Prevalence of HBV is divided into three categories for the sake of convenience High :>8% Asia-Pacific region

Intermediate: 2-8% India, Indonesia, Malaysia and Singapore

Low:<2% North America, Europe. Migration from high prevalence area to low prevalence area is an important factor as most carriers residing in this region were born in endemic regions.[9]

The prevalence of HBV infection is heterogeneous across the world, with an intermediate to high prevalence in our Asia-Pacific region, which contains three-quarters of chronic HBV-positive subjects worldwide.

The Western Pacific region (World Health Organization defines it as 37 countries including China, Japan, South Korea, Philippines, and Vietnam) accounts for almost 50 % of all chronic HBV infections across the world, although it has less than one-third of the world's population.[10]

India falls in the intermediate group. Our country has a prevalence of 2% to 4% in the general population but higher prevalence is seen in high risk populations like professional blood donors, health care workers and patients with chronic renal failure on hemodialysis, thalassemia and hemophilia.[11] Another study also showed similar HBsAg seroprevalence which is reported by World Health Organization (WHO) statistics, in the intermediate zone.[12]

Epidemiology of hepatitis B

ROUTES OF TRANSMISSION

Transmission occurs by parenteral routes including blood transfusion, unsterile injections, surgical equipment, needle prick injuries, sexual exposure and mother to child (vertical) transmission. The transmission in India is thought to mostly occur horizontally during early childhood by close physical contact unlike East Asia where the transmission is mostly vertical. The prevalence of HBsAg has been found to be 2.25% in the below 5 years age group in our country. Breast feeding does not increase the risk of transmission.[13]

In India, seroprevalance of HBsAg was found to be 0.9% in ante-natal women. The risk of vertical transmission increased with HBeAg positivity and increased HBV DNA levels. Both modes, vertical transmission during intra-partum period and horizontal transmission during peri-natal period and early childhood are important means of transmission of this infection in our country.[14] Horizontal transmission by a nonsexual close household contact or among children, from one child to another is also documented.[15]

Even when children born to carrier mothers were immunized actively as well as passively within 24 hours of birth, it has been found by molecular studies that the virus uses mutational tactics like sequential variation in antigenic region to escape detection by T cells and B cells. These findings stressed the need for administration of booster doses or a more effective vaccine for children born to HBsAg positive mothers.[16]

HBV is efficiently transmitted by sexual contact. Approximately 25% of the sexual contacts of a HBV infected person also test positive for the virus.[17]

The primary risk factors are unprotected sex with an HBV infected partner, mainly unvaccinated MSM and heterosexual individuals, with multiple sex partners or contact with sex workers .HBV genotype A was most commonly found in MSM candidates.[18]The increase in the presence of selective strains among homosexuals highlights the need of immunization of high risk population in America.[19]Tattooing is found to be an important means of transmission in our country.[14]

It has been found that infection control measures breaches, such as administration of drugs from multi-vial compounds and capillary blood sampling, are the most common routes for patient-to-patient transmission of this virus.[20]

In developing countries, 33% of the 16 billion annual injections administered worldwide are unsafe and cause approximately 20 million new Hepatitis B infections. [21]

Unsafe injection practices are rampant in our country and other developing nations. Reuse of syringe, needle or both is responsible for 16 million hepatitis B infections per year across the world.[22]An explosive outbreak of hepatitis was reported in Haryana in 1997 due to unnecessary and unsafe therapeutic injections.[23]

In Northern Germany, where the prevalence rate is very low (0.3%) and hepatitis B is a notifiable disease, Hepatitis B outbreak was reported in a nursing home which was associated with reusable lancet devices for blood glucose monitoring, in 2010.[24]

Simone Lanini *et al*. analyzed 30 papers which reported 33 outbreaks which included 471 patients along with 16 fatal cases and gave the following statistics. Dialysis units were responsible for 30.3% outbreaks, Medical Wards-21.2%, Nursing Homes-21.2%,Surgery Wards-15.2% ,Out- patient clinics-12.1% and the modes were Multi vial

drugs-30.3%, non-disposable blood sampling devices-27.2%, Endo myocardial Biopsy procedures– 9.1% and Multiple deficiencies in the application of the standard precautions-9.1%.[20]

Health-care professionals are at high risk during exposure prone procedures.

These health care workers become HBV carriers in 5-10% cases and are potential source of risk to the patient. Transmission rate from HBeAg positive doctor to patient is 24% during cesarean section and 5.5% to 13% during high risk procedures[7].

The possibility of infection by HBsAg negative HBV positive carrier cannot be ruled out during blood donation or liver transplantation.[25] Occult HBV infection and window period infections, to a lesser extent, are risk factors for transfusion transmitted HBV infection.[14]

A HBV NAT assay, which is highly sensitive, with a threshold much below 10 IU/ml is valuable in blood donor screening and in future, may be the only test for it in developed countries.[26]

Triplex nucleic acid testing detects infectious HBV along with HIV and HCV, during the window period before seroconversion has occurred[27]

Hemodialysis patients are at a high risk of infection.[15]

Low level persistence

HBV genome is retained by a major proportion of recovered patients in their liver. They continuously stimulate a cytotoxic T-cell response. This may be the only marker of previous infection by Hepatitis B virus. When the T cell immunity breaks

down, there may be reactivation of HBV replication .This usually occurs after organ transplantation or during cancer chemotherapy.[2]

Male sex and genotype C are independent risk factors for reactivation of virus.[28]

Even a good level of anti-HBs does not give total protection against reactivation as escape mutants may be present. Immune-suppression is very important in these cases as its withdrawal will lead to proliferation of memory T cells which will severely attack the infected liver. Hence, ideally all patients are to be screened before immunosuppression, for HBsAg as well as for occult HBV infection by Polymerase Chain Reaction assay.

HBV DNA levels > 10^5 copies/ml is a very important risk factor for HBV reactivation and lowering it by administration of nucleoside analogues should be considered before venturing into autologous hematopoietic cell transplantation in patients with hematologic or oncologic problems who are positive for HBsAg.[29]

Dose of infectious virus and mode of inoculation in the new host also affect the outcome of transmission. While intravenous infection is the most efficient means of transmission, mucocutaneous route is the least efficient means.

LIVER AS THE TARGET

Hepatocytes and bile duct epithelial cells originate from a common progenitor during embryonic life, so they are closely related and are unique to liver.

Hepatocytes, which are the major cell type in liver are the targets of infection by liver tropic viruses like HBV.

Bile duct epithelial cells, a subset of cells in the pancreas, kidney and lymphoid system may also harbor the virus.

As hepatocytes are normally long lived and usually non dividing, this, in combination with a stable interaction between the virus and the host, ensures the persistence of an infection in absence of a strong host immune response[30]

NATURAL HISTORY OF CHRONIC HEPATITIS B

There are three phases which result from the dynamic interplay between the virus and the host. Viral factors include the level of replication, and the host factors include gender, alcohol consumption, infection with other viruses, and the extent of immunosuppression.

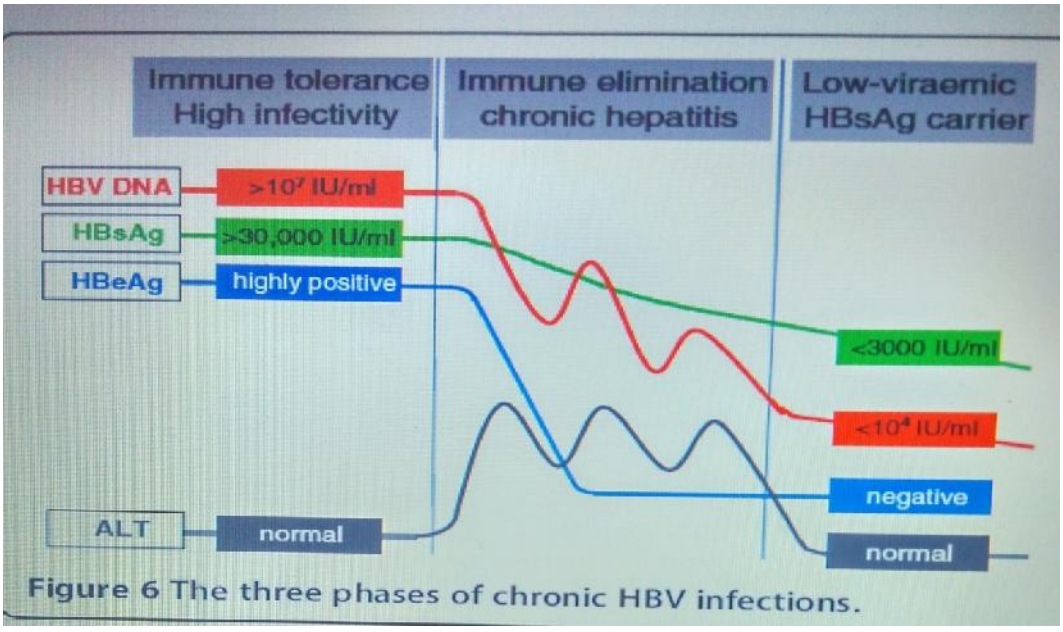
Phase 1 Immunetolerant phase: There is circulating HBsAg, HBeAg, and high level of HBV DNA. There is minimum immune response against the virus, so there are very little elevations of serum aminotransferases and very less inflammation in liver. In cases of perinatal infection, during this phase, (which may last for decades), there are very low rates of spontaneous seroconversion to anti HBe . The cumulative rate of spontaneous HBeAg clearance is estimated to be 2% in first 3 years of life and only 15% after 20 years. In contrast, in immune competent adults, this phase is typically present only during incubation period.

Phase 2 Phase of symptomatic acute hepatitis B: During this phase, there is a decrease in HBV DNA Level and an increase in immunity, accompanied by increased aminotransferases and inflammation in the liver. In this period there is augmentation of both innate and acquired HBV immunity, causing cytolytic destruction of hepatocytes. In

adults, this is the period of symptomatic acute Hepatitis B, although this phase may last for decades if the immune response is not strong enough to lead to virus clearance.

Phase 3 Phase of seroconversion: This third phase heralds the conversion from HBe antigenemia to anti HBe which is followed by reduction in virus replication and a decrease in aminotransferases. However, both HBsAg and low level of HBV DNA persist and that is the reason this phase is labeled as inactive carrier state. Seroconversion is marked by the entry of HBcAg specific CD4+ and CD8+ T cells in the circulation.

After the development of anti HBe, the third phase of illness, non-replicative phase begins. Termination of virus replication is associated in most patients with histological regression of inflammatory activity. The prognosis for healthy carriers is generally good in absence of cirrhosis, although these patients, if immunosuppressed, can have a reactivation of replication.



Three Phases of Chronic Hepatitis B Virus Infections

EMERGENCE OF HBV VARIANTS DURING A CHRONIC INFECTION

HBV is under selection pressure from the immune system and HBV variants may emerge over the course of a chronic infection. These variants generally facilitate immune evasion and have been detected throughout the viral genome. During the course of natural history, the mutants that are HBeAg negative are the most studied. These variants harbor mutation in the pre-core region and/or in the basic core promoter region. When such mutations occur, infections are characterized by presence of HBsAg, absence of HBeAg, and HBV DNA levels that are fluctuating, usually greater than 2000 IU per ml. This form of chronic hepatitis is associated with a high risk of liver fibrosis, cirrhosis and HCC. Also, unlike HBeAg-positive infection, treatment end point is difficult to state,because HBeAg seroconversion cannot be used as a surrogate marker for the cessation of virus replication.

• Generation of HBV variants can be by point mutations, small deletions or insertion, and by splicing events. HBV reverse transcription process is very inaccurate and all possible mutations may be created in a very short time. It is estimated that a chronic carrier who is highly viremic generates ca. 1013 new viral genomes per day. At an error rate of 10^{-5}, 108 mutants may be generated in a day. Any kind of selection causes a great variety of variants, whereas in quasi-immunotolerant state, one genome species gets strong favors. Breakdown of immune tolerance is indicated by disappearance of HBeAg and this leads to a rapid accumulation of some viable and some non-viable mutants. The main targets are

- The preS1/S2 junction causing a loss of MHBs

- The binding sites for transcription factors in the core promoter and enhancer region are altered, which leads to less HBeAg production and more replication.
- Inactivation of the pre-core region or
- Deletions in the core protein

The type of mutation leading to a HBeAg loss differs with the genotype. Immunosuppressed patients display X protein deletions. All these mutations supposedly develop under the only selection pressure, of host`s own immune system. Active or passive immunization may alter the balance and select for escape mutations in the HBsAg antigen loop. Treatment with nucleoside or nucleotide analogues usually selects for mutations in the reverse transcriptase domain of the HBV DNA Polymerase, which also become overt in the S domain. Often many types of selections come together which lead to complex combinations of mutations. Usually, heavily mutated variants are commonly found in late stage infection or HCC[31].

The most common pre-core mutant virus has a point mutation at nucleotide 1896 (A 1896) from G to A due to which, a stop codon 28, is created which abolishes the synthesis of HBeAg. However, pre-core and core mutations occur in both, HBeAg positive as well as HBeAg negative hepatitis. The clinical significance has to be defined in the perspective of antiviral therapy.[32]

In chronic hepatitis B patients, there is an increase in ´a` determinant variability which suggests a selection of HBV immune escape mutants during chronic carriage of virus.[33]

Some chronic carriers have a spontaneous clearing of HBsAg and it has been found that rs9277535 non GG genotypes in HLA-DPB1 region have a higher chance of spontaneous HBsAg seroclearance in chronic carriers.[34]

Genetic factors in the host like polymorphisms in interleukin-18 gene, Tumor Necrosis factor, α promoter polymorphisms and HLA-DP gene variants are linked to the persistence of HBV.[15]

OCCULT HBV INFECTIONS

Occult infection is characterized by the detection of HBV DNA by PCR in the serum of patients in whom serum HBsAg assays are negative. In patients with liver disease of unknown etiology, occult HBV infection can be up to 30 %. In such cases, the HBV genome is replication competent in tissue-cultures. Viral suppression observed in vivo is because of epigenetic control of intrahepatic ccc DNA. This ccc DNA is the molecular basis of occult infection because of its ability to persist in the nuclei of hepatocyte[35]. Significant number of HBsAg-negative patients with HCC is reported to have occult HBV infections. It is possible that some infections diagnosed as occult are so because they escaped detection due to mutations in S gene that prevented detection by the serological assay for HBsAg. In such patients, HBV DNA titersare usually very high.

The key mechanism in occult infection is the integration of HBV DNA in the core genome. The disruption and rearrangement of genes during this integration can cause

1. Loss of HBsAg from serum
2. Reduction in the production of virions
3. Loss of detectable level of HBV DNA in serum

Vitamin.D3 and the VDR, which regulate several cytokines, are important determinants of HBV response. Polymorphism in the VDR gene is associated with outcome of chronic HBV infection. HBV DNA level differences and loss of HBeAg are also linked to VDR genotypes[15]

The diagnosis of occult hepatitis B requires more sensitive detection of HBsAg in blood(<0.1ng/ml) and HBV detection(<10IU/ml). Detection of HBV DNA in liver biopsy is advocated and for this, frozen sections are preferred over formalin fixed ones but this approach is unsuitable.

The target population for occult hepatitis B screening includes patients with liver disease, those with primary malignancy and patients undergoing haemodialysis[35].

According to evidence, chronic consumption of alcohol may down regulate expression of HBV antigen and may partly account for HBV DNA positive test in HBsAg negative alcoholic patients.[25]

VIRAL PROTEINS AND PARTICLES

The 22-nm particles are the most numerous amongst the three particulate forms of HBV. They are believed to represent excess viral envelop protein and are seen as spherical or long filamentous forms which are antigenically similar to the outer envelope protein of HBV. There are large, 42 nm, double-shelled spherical particles, which represent the complete hepatitis B virion. The envelope protein, present on the outer surface of virion and on the smaller spherical and tubular structures is known as hepatitis B surface antigen. It is the product of S gene of HBV.

Sub determinants of envelope HBsAg include a common group-reactive antigen, *a* , which is shared by all HBsAg isolates and one of several subtype –specific antigens- *dor y,* w or *r* – along with other specificities.[4]

These are associated with variations in single aminoacid, at positions 122 and 160 respectively.[36]

Hepatitis B isolates fall into one of eight subtypes and ten genotypes(A-J). Distribution of genotypes and subtypes varies according to Geography; genotype A (corresponding to subtype *adw*) and D*(ayw*) predominates in the United States and Europe, whereas genotypes B(*adw)* and C*(adr)* are more common in Asia. Clinical course and outcome do not depend on subtype, but genome B is supposed to be associated with less rapidly progressive liver disease and cirrhosis and a lower and later chance of hepatocellular carcinoma than genotype C or D. Patients with genotype A are more likely to clear circulating viremia and to achieve HBeAg and HBsAg seroconversion, spontaneously as well as after antiviral therapy. Also, certain genotypes favor pre-core mutants.

Upstream of the S gene are the pre-S genes,on which the pre-S gene products are coded which include receptors on the HBV surface for polymerized human serum albumin and for hepatocyte membrane proteins. The pre-S region consists of pre-S1 and pre-S2.

The synthesis of HBsAg gene products depends on the location of initiation of translation.

HBsAg (Major protein) is the protein product of S gene.

Middle protein is the product of S region and the adjacent pre-S2 region.

Large protein is the product of pre-S2 and S region.

42 nm virions have more of large protein compared to smaller particles of HBV. During HBV infection, both, pre-S proteins and their antibodies can be detected.

The 42-nm virion contains a 27 –nm nucleocapsid core particle. C gene codes for Nucleocapsid protein. The antigen present on the surface of the nucleocapsid core is *hepatitis B core antigen* (HBcAg). Anti-HBc is its corresponding antibody.

The C gene has two initiation codons. One is present in the pre- core region and another, in the core region. If the translation process begins in the Pre-core region, HBeAg is produced .It is a very important nucleocapsid protein which is soluble and nonparticulate. It has a signal peptide by which it can bind to the smooth endoplasmic reticulum. This smooth endoplasmic reticulum is the secretory apparatus of the cell which mediates the secretion of HBeAg into circulation.

If the translation begins in core region, HBcAg is formed. It does not have a signal peptide and hence is not secreted. It forms nucleocapsid particles, which eventually contain HBV DNA. HBcAg particles are retained in the hepatocyte, where they can be detected by immunohistochemical staining. They are encapsidated in an envelope of HBsAg and then are exported out. Naked core particles do not circulate in the serum.

The secreted nucleocapsid protein, HBeAg provides an easily detectable, qualitative marker of HBV replication and its relative infectivity[4].

HBeAg positivity is associated with an increased risk of hepatocellular carcinoma[37]

Nucleocapsid core also has is a DNA polymerase, which choreographs replication and repair of HBV DNA.

HBsAg –positive serum containing HBeAg is more infectious and associated with the presence of hepatitis B virions (and detectable HBV DNA) than HBeAg -negative or anti-HBe-positive serum. HBsAg positive mothers who are HBeAg -positive transmit hepatitis B infection to their offspringalmost invariably (>90%),whereas HBsAg positive mothers with anti-HBe infect their offspring rarely (10-15%).

In early stages of acute hepatitis B,HBeAg appears transiently;its absence may be seen as an evidence of clinical improvement and resolution of infection. Presence of HBeAg in serum for more than the first three months of acute infection may predict the development of chronic infectionand the presence of HBeAg during chronic hepatitis B is associated with persistent viral replication, infectivity, and inflammatory liver injury(except during the perinatally transmitted HBV infection in early decades).

The P gene, which is the largest of HBV genes codes for HBV DNA polymerase; this enzyme contains both DNA-dependent DNA polymerase and RNA-dependent reverse transcriptase activities. The fourth gene, X, codes for a nonparticulate protein, *hepatitis B x antigen*, (HBxAg), which has the capacity to Trans activate the transcription of viral and cellular genes. In the cytoplasm, HBxAg effects calcium release, which activates signal –transduction pathways. These pathways cause stimulation of viral reverse transcription and HBV DNA replication.[4]

HBx is known to be oncogenic. The HBx/CDC42/IQGAP1 signaling pathway may play an important role in carcinogenesis mediated by HBx.[38]The truncated x protein contributes to hepatocarcinogenesis by upregulating *RAS* and *MYC*.Many other mutations in the x gene like G1896A,G1899A,the pre-S1 deletion and the pre-S2 deletion are associated significantly with the development of HCC.[36]

GENOME STUCTURE AND FUNCTION

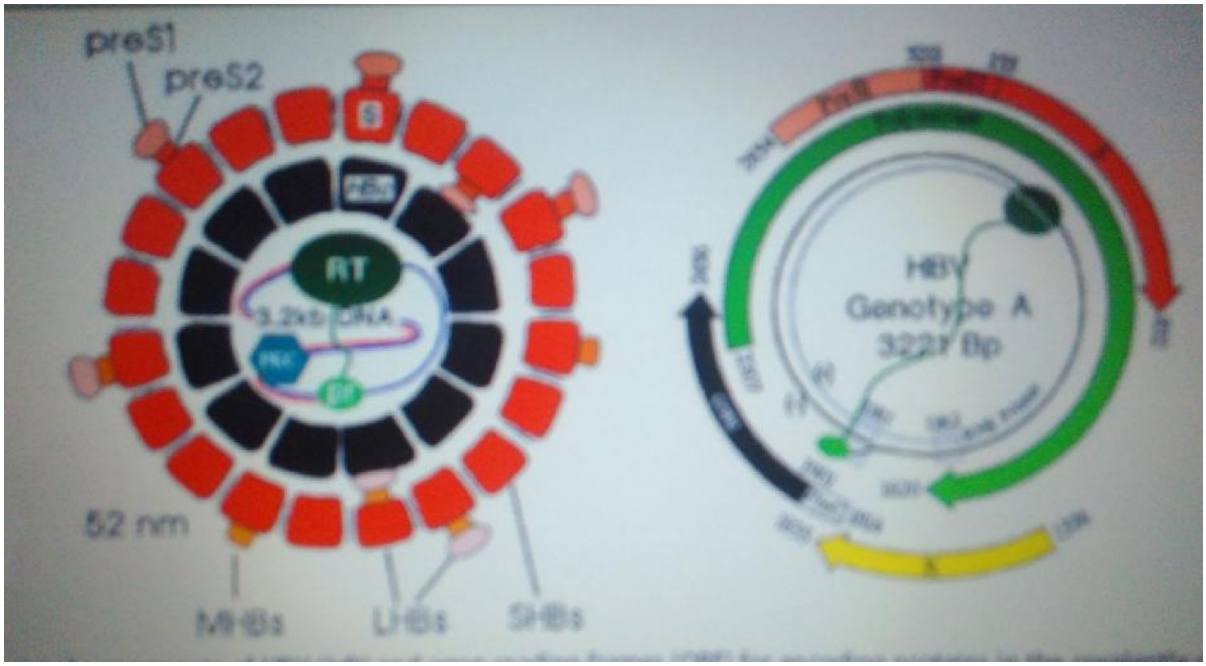
The HBV genome consists of a circular, partially double stranded DNA. It has a longer strand of negative polarity which is ca.3200 nt long. It is covalently bound to the viral polymerase. The polymerase presents the hydroxyl residues of tyrosine as the acceptor for phospho di ester linkage to the nucleotide of the negative DNA strand. The 5' and 3'end of this DNA strand are not linked covalently. The circular structure is because of base pairing of the negative with the positive DNA strand. The positive DNA strand has a defined 5' end at the upstream of the 3' end of the negative DNA strand. The 5' end is linked to a RNA oligonucleotide. The length of the positive DNA strand varies between 1100 to 2600 nt, depending on the progress of the viral polymerase. Therefore, a single-stranded region is left and this space can be partially filled in vitro by the viral polymerase.

The physical structure of the DNA has been confirmed by the cloning and sequencing of HBV DNA. The genome of hepadnaviruses contains four open reading frames (ORF), on the negative DNA strand which are conserved and partially overlapping. Thus, the coding capacity is increased and the capacity of3200 nt corresponds to 5 500 nt (had ORFs been arranged in linear manner). The ORFs code for:

- The core protein and an additional pre core region containing a signal peptide sequence; this additional precore peptide causes HBe formation.
- The DNA polymerase
- Set of surface proteins which is nested
- A protein, X protein (HBx), which was thought to be absent in the avian hepadna viruses but recent studies have proved otherwise.

The ORF expression is controlled by four promoters, enhancers 1 and 11, glucocorticoid-responsive elements (GRE), and a negative regulating element (NRE). Termination of transcription is coded by a single poly adenylation signal and hence all viral mRNAs share a similar 3' end.

Additional regulatory elements are present on the level of mRNAs that are transcribed. Post-transcriptional regulatory element (PRE) is present, which cause suppression of splicing of the transcribed RNA. The pregenome has signals which support reverse transcription and the formation of DNA which is circular. This includes the Є signal. This signal is required for binding of the viral polymerase and eventually initiation of reverse transcription and two direct repeats (DR),which are termed DR1 and DR2. The minus –strand DNA, which is the formed from reverse transcription of the RNA pregenome, constitutes the RNA pregenome, the M region, responsible for the correct translocation of the polymerase to the site where subsequent plus-strand DNA synthesis begins. Circularization of the minus strand requires an extra element as the terminal redundancy of the minus strand (r) and two domains, 5E and 3E.[39]



STRUCTURE OF VIRUS

Viral Life-cycle: An Overview

Attachment and Entry

Entry in the nucleus and Formation of episomal Viral DNA

Transcription and Translation

Assembly of the Viral Nucleocapsid and Maturation of Genome

trafficking of Progeny viral Capsids

Transport to the nucleus

Envelopment and secretion

ATTACHMENT AND ENTRY

HBV infects only humans; hence it is adapted to hepatocytes which are fully differentiated. The preS1 protein sequence 10-36 (21-47 in HBV genotype A) is important for attachment of HBV to human hepatocyte membranes, and this binding can be blocked by competing peptides or by antibodies that are active against the sequence.

The preS2 domain of HBV binds with a modified form of human serum albumin. This modified human serum albumin is the mediator in the binding of preS2-containing HBsAg particles including HBV to human hepatocyte membranes, but it is not proven that this phenomenon contributes to infection. In the initial stage HBV reversibly attaches to host cell surface proteoglycans and this is later followed by the process which involves more specific receptors and after internalization which is endocytosis-mediated, the virus fuses with the cellular membrane compartment, probably in an endosomal compartment.

Host specificity and tissue tropism are because of specific receptor recognition. The initial attachment step is mediated by heparin sulfate proteoglycans. The S and preS1, but not the preS2, regions of the virus play a significant role in HBV infection. The pre-S1 region is predominantly involved.[39]

Sodium Taurocholate Co-Transporting Polypeptide (NTCP) has been found to be Bona Fide HBV Receptor. This is a major landmark in the study of molecular biology of Hepatitis B virus.This receptor is present on the basolateral membrane of hepatocyte. It plays an important role in hepatic influx of bile salts from portal circulation. The gene coding for NTCP is the SLC10A1 gene and is located on chromosome 14. It is now a target for new anti HBV agents. However there are other additional cellular factors which are crucial to HBV entry.[40]

All hepadnaviruses, contain a short hydrophobic sequence at the amino end of their S domain, which is similar to fusion peptides of many enveloped viruses. This sequence has an important role in membrane fusion.

NUCLEAR ENTRY AND FORMATION OF EPISOMAL VIRAL DNA

Liver cells are highly differentiated and mostly non-dividing cells. The hepadnaviral genome hasto migrate through the cytoplasm towards the nuclear pores, later followed by the passage through the pore. The relaxed circular (rc) DNA has to be released from the capsid for conversion to the cccDNA by cellular enzymes. HBV capsids can pass through the nuclear pore as entire particles as they are small enough.

After release from the capsid, the double stranded DNA genome is converted to cccDNA by cellular modifying enzymes. Formation ofcccDNA is the first marker of successful infection. Later, there is formation of replicative intermediates.

The formation of ccc DNA happens in the following steps:

- There is removal of covalently bound polymerase and the 5`terminal redundancies from the negative strand are removed.
- RNA primer is removed
- Positive DNA strand is completed
- There is linkage of 5`and 3` ends of each strand by means of DNA ligase.
- Viral as well as cellular polymerases are required to fill up the positive DNA strand.

TRANSCRIPTION AND TRANSLATION

All mammalian hepadnaviruses genome has one polyadenylation signal TATAAA. Upon Cytokine induction, HBV RNA is downregulated post transcriptionally. Stabilization of the HBV transcripts counteracts the decrease of HBV mRNA. The turnover of HBV RNAs depends less on the primary nucleotide sequence than on structural features.

The translation of the first reading frame is most efficient like in most eukaryotic mRNAs. So, the first encoded ORF ex, HBe-,preS1, pre S2/S- and X-RNA decide the names of different RNAs. There are two sets among these RNAs.

1. RNAs, of length smaller than the genome, that encode for X and surface proteins

2. Supergenomic RNAs that are longer than the genome and code for HBe, core and polymerase.

The ca.700 nt long HBx RNA begins at two initiation sites and has many binding sites for liver specific transcription factors making a tissue specific transcription of this RNA a strong possibility but it is expressed on non-hepatic cells also.

The HBV genome is transcribed on hepatic as well as non hepatic cell lines.When the first AUG is used, MHBs are expressed. Mostly second AUG is also used resulting in expression of the small surface protein, SHB. Its preS2/S promoter has very active SP1 binding sites and therefore SHBs can be expressed on a wide variety of cell lines and tissues.

However, the LHBs-encoding mRNA has liver specific transcription, is of ca 2.4 kb and requires factors HNF1 and 3.This protein expression is essential for secretion and envelopment of the virus.

Super genomic mRNAs have two sets which are ca.3.3 kb long. Their transcription is liver specific and requires binding sites for nuclear hormone receptors, such as HNF4, retinoid X receptor and peroxisome proliferator –activated receptor alpha. The transcription of two classes of RNAs is initiated by the core/ HBe promoter which have totally different biological functions.AUG start codon is present only on pre C region of 5`end of longer RNAs, which is present in frame with AUG of core reading frame. Hence there is a difference of 29 additional aminoacids between its product and the core protein at the amino terminal end. It is called pre C which has a hydrophobic signal sequence for being secreted into the ER. HBe antigen is the resulting product.

Expression of hepadnaviral polymerase does not occur by ribosomal frame shifting but by de novo translation initiation.

The nucleotide sequence in the core promoter/enhancer decides the ratio between pregenomic and preCore HBe m RNA. Mutations that occur from A1762 to T and G1764 to A enhance pregenome expression and replication and reduce HBeAg expression whereas HNF 3β inhibits pregenome transcription and replication, favoring expression of HBe mRNA.

Two virus encoded transcription factors, apart from cellular transcription factors, regulate HBV gene expression.

HBx activates HBV replication as it activates tyrosine kinases and calcium signaling. The second transcription activating element acts only when it is situated at the cytosolic side of endoplasmic reticulum and is located in the pre-S2 domain. HBV replication requires raf/MEK signal cascade which is activated by PreS2.

ASSEMBLY OF THE VIRAL NUCLEOCAPSID AND GENOME MATURATION

The viral polymerase and the epsilon (ε) signal mediate the specific encapsidation of pre genomic RNA, therefore mutations in polymerase gene will obviously affect this phenomenon.

Encapsidation is supported by only the 5‘terminal ε. All hepadnaviruses have conserved the structure of the base–paired stem-loop that is formed by ε. Additional sequence elements increase the efficiency of pregenome encapsidation by viral polymerase, even though ε alone is sufficient, which probably explains how, in the super

genomic and sub genomic RNAs, the viral polymerase discriminates 5‘terminal ε from 3‘terminal ε. The ribosomes on the HBe RNA make the HBe mRNA nonreactive by preventing the folding of ε or its interaction with the polymerase. As the pregenomic RNA has to be scanned by 40S ribosomal complex for the beginning of core protein synthesis, only the 80S translating ribosomes can displace the ε-bound polymerase.

A protein kinase is also encapsidated in core particle which phosphorylates serine residues in between the arginine clusters in the core protein at its carboxy- terminal. This terminal is multifunctional and has domains which are essential for RNA packaging and maturation of the genome. Hence, there is almost complete inhibition of maturation of genome even by point mutations of the protein kinase target sequences.

There are many complex events by which linear RNA pregenome is converted in to circular double stranded DNA.

The polymerase begins at the bulge of the ε signal. After first four nt which are encoded by the bulge are added, the oligonucleotide polymerase complex dissociates and reattaches with the complementary sequence to the 3`terminal DR1. Additional factors like the primary as well as the secondary structures of the surrounding RNA, sequence of DR1 and the distance between it and the initiation site, are involved. As in Retroviruses, the catalytic center which is required for the polymerization reactioncontains the typical YMDD pattern. It is also common that DNA-RNA hybrids result.

At DR2, the 3`end of the RNA oligonucleotide is used as DNA synthesis primer by the polymerase. As the polymerase and the 5`end of the minus strand are covalently

bound, the strand has to form a loop.Also, the space between phosphodiester linked Tyr in the priming domain and the polymerase catalytic center is important.

HBV genome in core particles is circular predominantly, therefore the polymerase can bridge the gap between 3`and 5`end of negative DNA strand. There are many more requirements for circularization.

The virion contains a partially double-stranded DNA with the heterogeneous3`ends of the plus DNA strand leaving a gap of ca.800 nt in the single strand. Few virions have a linear double stranded DNA genome and they are also infectious.

TRAFFICK OF PROGENY CAPSIDS

Transport to the nucleus

For viral persistence, the pool of intranuclear HBV genomes has to be restored permanently. It is possible by

- New infection of the liver cells by virus
- Re-entry of the viral DNA after being released. This is the more important of the two and it occurs in the beginning of the infection.

The chronic infection is established on cellular level and the virus does not appear in the serum and hence cannot be neutralized by circulating antibodies.

In vitro systems to study how the viral genome reaches the nucleus show that it is transported inside the core particles, using the microtubule transport system of the cell. From the perinuclear region, there is active nuclear import. To interact with import

factors, a classical localization signal (NLS) is displayed by the core proteins on the capsid surface. This requires a structural change.

After the establishment of the nuclear import pathway, importin β mediates the binding and the journey through the pore into the nuclear basket. It is exclusively at this site that the disintegration of the core particles takes place and the viral DNA is released into the karyoplasm.

ENVELOPMENT AND SECRETION

Core particles get enveloped into surface proteins followed by virus secretion. LHBs and SHBs are required for the assembly of HBV. The sequences which are present before amino acid 108 of pre S are required for assembling HBV. There is a highly conserved pre-S1 region between Arg103 and Ser 124, which is essential.

The pre-S1 domain acts as site of attachment to the cell surface during infection. Therefore it has to be accessible on the surface of the particle.

Envelopment negative core mutants have been identified which are clustered at two sites on the surface of the capsid.

HBV and HBs particles, for their budding and assembly require the presence of most of the cysteine residues. There may be a coil- to- coil interaction which contributes to the morphogenesis.

The maturation of HBs particles occurs in a pre-Golgi compartment. HBsAg 20 nm spheres are secreted constitutively. HBs-filaments may be held back in Endoplasmic

reticulum if they contain more LHBs than SHBs. Only the cells that have a well-balanced proportion of core protein, polymerase, SHBs and LHBs can assemble and secrete HBV.

Some liver cells in HBsAg carriers over express LHBs and so have a dilated endoplasmic reticulum. This gives them a ground glass appearance when viewed under light microscopy. These cells do not participate in HBV production and are found in low viremic healthy carriers.[31]

Chronic HBV infection was associated with accelerated ageing of hepatocytes and with hepatocyte G1 cell cycle arrest. It implied that HBV induced cellular senescence .HBV replication was restricted to biologically younger hepatocytes.[41]

HBV suppresses the total T cell population, specifically the cytotoxic T cell population, in the peripheral circulation. This is more obvious in patients with higher viral load.[42]

PREGNANCY AND HBV

Chronic HBV carriers had a minimally increased risk of preterm birth and low birth weight but the risk was pronounced in women with HBeAg positive status. They also had an increased risk of Gestational diabetes mellitus.[43] Inactive hepatitis B infection does not increase the risk of complications during the pregnancy like PROM, preeclampsia, eclampsia, gestational hypertension and antepartum hemorrhage.[17]

HBeAg is important for HBV to suppress $CD8^+$ T cell response and persist in the host.

In a study of mouse model, it was found that HBV which is carried by the mother impaired $CD8^+$ T cell responses to HBV in her baby, which resulted in HBV persistence. This impairment of response was mediated by hepatic macrophages. The maternal HBeAg predisposes the hepatic macrophages to support the persistence of HBV by

- Up regulation of inhibitory ligand PD-L1 and alteration in polarization when stimulated again by HBeAg.

Depletion of hepatic macrophages caused HBV clearance in the offspring.

There is a prospect in which macrophages are targeted to treat chronic HBV patients.[44]

HLA gene variants are associated with susceptibility to vertical transmission.

Apart from other factors like HBV structure, DNA level, placental barrier, immune state of mother, genetic makeup of the new born,HLA-DRB1*07 is associated with infant susceptibility to intrauterine infection with HBV.[45]

Maternal HBV status may be a risk factor for miscarriages[46].The parturients who were HBeAg positive before child birth,70% of them did not have HBeAg clearance from serum even 5 years after delivery. Aggressive follow-up is required in such cases.

There is a downward trend in HBV carrier status in pregnant women since universal immunization.[47]

In our country, seroprevalance of HBV was found to be 0.9%. The risk of vertical transmission increased with HBeAg positive status and high viral DNA levels Vertical

and horizontal transmission during pregnancy and early childhood are major routes of transmission in our country.[14]

Many changes take place in the maternal immune system to prevent the rejection of fetus during pregnancy. After delivery, all these changes revert back and therefore the post-partum women are to be closely monitored for hepatitis B flares.

Women with HBV infection are encouraged to breastfeed after the infant has received immune prophylaxis at birth.[48]

An infant whose mother is both HBsAg positive as well as HBeAg positive and who has not received any active or passive immunization, has 70-90% chance of being HBsAg positive. He has upto 90% possibility of becoming a chronic carrier.

There is no statistically significant difference in prevalence of HBV infection in children of different age groups below 5 years of age. Hence children in this age group are affected via vertical transmission .This is responsible for majority of the chronic carriers in our country. Universal immunization is advocated to decrease the carrier pool and should preferably begin at birth.[13]

DIAGNOSIS OF HEPATITIS B

SEROLOGY

This remains the most important tool in which, the immunoassay of viral antigen and its corresponding antibody is done. The presence of serological markers helps to predict the stage of disease, infectivity of patients and also helps to select the patients for therapy and later, monitoring it.

Molecular methods are fast gaining popularity as they directly detect viral DNA.

HBsAg

It is the key marker of Hepatitis B infection. It begins to appear in serum by 1-10 weeks after exposure and disappears in 6 months. Cutoff value for its detection is 3×10^7 particles/ml.

Anti-HBs

These appear after the corresponding antibodies have disappeared and persist through the life. Their presence is indicative of immunity acquired either by infection or by immunization.

HBeAg

It is the marker of viral replication. Its loss signifies clinical resolution with cessation of viral replication but it is not always so.

Anti-HBe

It is the corresponding antibody. Its appearance is also indicative of cessation of replicative activity but in cases of mutants, replication of virus occurs even in its presence.

Anti-HBc

It is the first IgM antibody to appear in the serum after infection of Hepatitis B virus. Being the first immunological sign of acute infection it is present throughout the infection and is a confirmatory marker for the diagnosis of HBV. When it is present in cases of chronic Hepatitis B it indicates re-activation of infection. It is the only marker

detected during the window period, between the disappearance of HBsAg and appearance of Anti HBs.

MOLECULAR METHODS

Detection and quantitation of HBV has become integral to the diagnosis and management of chronic Hepatitis B. Many types of molecular assays are available out of which polymerase chain reaction is the most preferred.

PCR is based on the principle of viral lysis, extraction and purification of DNA which is further amplified and then quantified.

It is highly sensitive and has excellent level of performance with a range of detection from 5-10 IU/ml up to 10^{8-9} IU/ml or more. However this is a dynamic parameter and hence, serial monitoring is mandatory. Sensitive nucleic acid testing for the precise quantitation of hepatitis B virus (HBV) is of paramount importance to decrease the probability of transmission through blood during blood donation especially if the donor is occult positive for HBV, and for monitoring patients on antiviral therapy.[49]

Data from REVEAL-HBV study (Risk Evaluation of Viral Load Elevation and Associated Liver Disease/cancer) demonstrated that incidence of HCC correlates with the viral load in serum Hepatitis B in dose dependent manner.

Relative Risk started increasing from HBV DNA levels of 2000 IU/ml up to HBV DNA levels of 2,00,000 IU/ml.

In ERADICATE-B study (Elucidation of Risk factors for Disease Control or Advancement in Taiwanese Hepatitis B carriers), elevated HBV DNA level, along with

HBsAg, was positively associated with development of Hepatocellular carcinoma in dose dependent manner when HBV DNA > 2000 IU/ml.

Data from REVEAL-HBV study and ERADICATE B study showed that HBV DNA, along with serum HBsAg , predicted the progression of disease including hepatic flares, HBeAg negative hepatitis, liver cirrhosis and hepatocellular carcinoma.[50]

The gold standard for the diagnosis of occult infection of HBV is the study of HBV-DNA extracted from liver or blood. DNA detection from liver biopsy is the best option as viral genomes persist in hepatocytes. Frozen samples are preferred for this test.[35]

Evaluation of relationship between HBV DNA levels and hepatic pathology is the current hot spot in the diagnosis and treatment of HBV.[51]

It is helpful in choosing patients for treatment and early diagnosis of drug resistant variants. However, the selection of rtA181T/sW172[*] and its emergence masks the diagnosis of resistance if only serum DNA levels are used for diagnosis. HBV polymerase sequencing or other genotype methods like line probe assay is required to diagnose drug resistance in such cases.[52]

It is useful in deciding the end point of treatment for CHB. For patients who are infected in early life, it should be suppression of HBV-DNA levels below the threshold of detection by PCR[53].

Most important application of viral DNA is in the diagnosis of HBeAg negative variants.

Co -infections.

HBV and other blood borne viruses like HCV, HDV and HIV have overlapping patterns of endemicity because they share their modes of transmission. Co –infections modify the natural history of disease and are associated with poorer prognosis than mono infection. It is important to protect those living with HBV against the other viruses by vaccination and behavior modification.[9]

Alcohol and co infection with hepatitis C & D are the most common environmental co-factors associated with liver disease progression in chronic HBV carriers.[54]

IMMUNIZATION AGAINST HEPATITIS B

Vaccine against Hepatitis B protects against it and its complications, which include liver cirrhosis and Hepatocellular carcinoma. It is the first vaccine against cancer, the first vaccine that protects against Sexually transmitted diseases and also the first licensed vaccine against a chronic disease.[21]

World Health Organization, in 1992, recommended that all countries should introduce Universal Hepatitis B vaccination for the immunization of infants and adolescents in their National Immunization Program by 1997.

Active immunization and passive immunization

Passive immunization

Human immunoglobulins, given within 24 hours of birth to the neonate, give protection for about 22 days and then their protective effect wears off.[6]Single dose of

HBIG is administered intramuscularly immediately after birth with active immunization at birth, after 1 month and then six months after the first dose.

Infant vaccination with a recombinant hepatitis B vaccine gives long-term protection against clinical disease and new chronic hepatitis B infection even if hepatitis B exposure is confirmed.[55]

Active immunization

Pre-exposure prophylaxis:

This is done in high risk groups that we have seen earlier. Active immunization is done. Adult dose consists of three vaccines 1ml each to be given intramuscularly in the deltoid muscle. Second dose is to be given one month after the first and third dose follows six months after the first one.

Post-exposure prophylaxis:

Single dose of HBIG is given in adults immediately or as soon as possible after the exposure, by intramuscular route. Later, active vaccination is done as per schedule. Persons who are at the risk of sexual transmission can be passively immunized within 14 days of exposure. In hemodialysis patients, booster dose is recommended in certain situations.

HBsAg contains a group specific *a* determinant that is common to all subtypes of Hepatitis B. This determinant, that has HBsAg codon 121-149, is within major hydrophilic region and is assumed to have a two loop structure.

It is the major target for antibodies which develop either after natural infection or following Immunization.[56]

Hepacare is a third generation DNA vaccine that contains Pre-S1,S2 and single antigenic components of HBsAg, both adw and ayw. All three components are glycosylated. It is presented as an Aluminium hydroxide adjuvant preparation of purified antigen in 1ml of isotonic saline.[55]

Childhood chronic HBV infection prevalence has been significantly reduced in Vietnam due to vaccination. Further strengthening of vaccination will be important for reducing chronic HBV infection prevalence of under 5 children to <1% which is a national and Western Pacific regional hepatitis B control goal.[57]

Decrease in Hepatitis B infection among vaccinated parturients reflects the effectivity and impact of Universal Hepatitis B immunization.[47]

The prevention of HCC by HBV vaccine is from childhood to early adulthood. Failure to prevent HCC is because of unsuccessful control of HBV infection in highly infectious mother as prenatal maternal HBsAg status is the most important factor in chronic HBV infection and hence the development of HCC.[58]

ELIMINATION AND ERADICATION OF HBV

As there are no reservoirs for this infection other than human-beings, an aggressive approach towards eradication may eliminate the virus and this being very difficult may at least bring down the prevalence levels drastically. Three pronged strategy is deviced.

- .HBV in the chronically infected has to be eradicated or effectively suppressed.
- All the routes of infection should be interrupted.
- All susceptible population should be immunized. Vaccination is extremely effective and universal vaccination is regarded as the most important step in the elimination and eradication of disease. However the economic burden of doing so is a major obstacle[59].
- Primary infant vaccination with a recombinant hepatitis B vaccine confers long-term protection against clinical disease and new chronic hepatitis B infection even when there is confirmed hepatitis B exposure.[55]

TREATMENT OF HEPATITIS B

The management of Hepatitis B is directed towards suppressing viral replication. Those patients who have a continued, high level HBV replication are at highest risk for HCC. Clinical trials focus on clinical end-points over 1-2 years like

- Suppression of HBV DNA to undetectable levels
- Loss of HBsAg and/or HBeAg
- Improvement in histological status
- Normal value of ALT,

These short term gains reduce the risk of hepatic decompensation, clinical progression and death.

Important drugs that are approved for treatment of chronic HBV are injectable interferons (IFN) α, pegylated interferon and agents like lamivudine, adefovir, dipivoxil, entecavir, telbivudine and tenofir.[60]

SURVEILLANCE FOR HCC

Risk of developing HCC may increase 100 times in chronic HBV carriers. This presses on the need for better surveillance measures of these people for early diagnosis and treatment.

HBV DNA fragments are integrated in the host chromosomal DNA. This occurs in the majority of HBV related HCCs. This has the potential to modulate the expression of the tumor suppressor genes and the oncogenes involved in carcinogenesis.

Molecular mechanisms which lead to occult HBV infection have a definite role in carcinogenesis.

Increased expression of APOBEC3B in HCC tissues gave a selective clonal growth advantage to pre neoplastic liver cells.[15]

Population of people with HBV Infection for Which Surveillance for Hepatocellular (HCC) is recommended or uncertain

RECOMMENDED

- Asian Male who is HBV carrier and is over the age 40.
- Asian female, HBV carrier over age 50.
- HBV carriers with a family history of HCC.
- HBV carriers with cirrhosis.
- Patients who are diagnosed to have high serum levels of HBV DNA and ongoing liver injury.

UNCERTAIN

- African Americans.
- HBV carriers in immune tolerant phase.
- HBV carriers with concomitant HDV infection, HCV infection, or nonalcoholic steatohepatitis.
- HBV carriers younger than age 40 (males) or 50 (females).

HBV DNA Levels and long term complications

Population based cohort studies in Asia have established that the serum HBV DNA level is the single best predictor of future progression to cirrhosis and HCC in people infected with HBV. In the prospective REVEAL-HBV natural history cohort study, more than 3600 untreated HBsAg carriers residing in Taiwan were followed for a period of more than 11 years. Of these, 60 % were males, 40% were older than age 50, 85% were HBeAg negative and 95% had normal serum ALT levels using standard reference ranges. The calculated relative risk for cirrhosis and HCC were shown to correlate with the level of HBV DNA on entry into the study when compared with a reference population of HBsAg carriers with undetectable serum HBV DNA by PCR assay. Even HBV DNA levels as low as 10,000 copies /ml(equivalent to 2000 IU/ml)were associated with a higher relative risk of cirrhosis and HCC. The relative risk was highest in persons with a serum HBV DNA level that was greater than100,000 copies/ml and intermediate in whom the serum HBV DNA levels decreased spontaneously from greater than 100,000 copies/ml at the time of enrollment to less than

10,000 copies/ml at the last point of follow-up. These data mean that both duration and level of viremia are important risk factors for the development of HCC. The data also suggest that suppression of serum HBV DNA levels, whether spontaneously or due to antiviral therapy, lowers the risk of HCC.[61]

A case control study done in China revealed that family history of HCC was a risk factor for HCC in the carriers of HBV. Carriers who did not have the gene for glutathione S-transferase M1 may have higher risk for HCC as there is heightened sensitivity to DNA mutation by Aflatoxin B1.[30]

MATERIAL AND METHODS

Type of Study: Cross Sectional Study

Source of data:

All patients attending the O.P.D/IPD of Shri B.M. Patil Medical College and hospital.

Study period : January 2016 to June 2017

Rationale for sample size[62]

The correlation between HBsAg and hepatitis viral load was found = 0.443

(Spearman rank test) Spearman`s rho

P=0.443

Considering probability of α errors = 0.05

And power of study = 80%

Formula Used: $N=\left[\left(\frac{Z_\alpha+Z_\beta}{C}\right)\right]^2+3$

$C=0.5*\ln\left[\frac{1+r}{1-r}\right]$ =0.476

Sample size =30

Statistical analysis includes:

Diagrams(Charts and Graphs)

Mean ± standard deviation

Correlation co-efficient

Inclusion criteria

HBsAg positive status for a period of at least 6 months in the person irrespective of sex

Exclusion criteria

- Patients with co-existent HIV, HAV or HCV
- Patients who have decompensated hepatic status, cirrhosis , hepatocellular carcinoma
- Impaired renal clearance S. Creatinine > 4 mg/dl
- Severe malnutrition, Auto-immune liver diseases and those who did not give consent

METHODS

The patients who are positive for HBsAg for at least 6 months were enrolled.

5 ml of blood was drawn with full aseptic precautions and 2 ml was put in purple capped (EDTA) vial and 3 ml in red capped (plain) tube.

For Hepatitis B surface antigen HBsAg

ELISA (Enzyme Linked Immunosorbent Assay)

For HBeAg

Automated Bidirectionally Interfaced Chemi Luminescent Immunoassay

Red capped vacutainer containing sample was centrifuged and serum was separated for study. It was labeled and stored at -20^{o} C.

ELISA FOR HBsAg:

Reconstitution of Reagents

Washing solution was diluted 1:20 in distilled water and homogenized.

All reagents and test samples were brought to room temperature

In each run , one well was assigned for the blank (A1), three wells for the HBsAg negative control (B1, C1, D1) and one well for the HBsAg positive control (E1).

100µl of the sample diluent was added in A1 (blank).

25µl of the sample diluent to the rest of the wells.

75µl of the HBsAg negative control was added in wells B1, C1 and D1.

75µl of the HBsAg positive control was added to well E1.

75µl of samples were added to wells G1 and so on.

50µl of conjugate was added to all the wells including the blank well (A1).

The wells were covered by a strip sealer after mixing and incubated at 37℃ for 60 minutes.

Sealer was removed and contents of the well were discarded into the waste disposal container. After that a minimum of 350 µl of washing solution was added to each well. It was also discarded after 30 seconds soaking time. The washing step was repeated 5 times.

50µl of color reagent was added to all wells including well A1.

The plate was covered with a black cover and the reaction was allowed to develop in the dark for 15 minutes at room temperature (20-30℃).

In the end 100 µl of stopping solution was added to all the wells and homogenized.

Color change to yellow was observed in positive samples while the negative samples remained colorless.

After wiping the plate bottom, optical density was read at 450 nm using 620 nm as the reference wavelength within 15 minutes of adding stop solution.

For HBeAg

Serum was sent to Thyrocare, Mumbai.

Fully Automated Bidirectionally Interfaced Chemiluminescent Immuno Assay was done.

Principle: when complimentary Antigen and Antibody are present together, they form an immune complex. CLIA estimates the level of such immune complexes by the use of labeled antibodies. Solid particles coated with antigen or antibody targeted are used. After incubation, substrate is added. This generates light ,the intensity of it being directly proportional to the labeled complex. It quantifies the analyte of intrest.

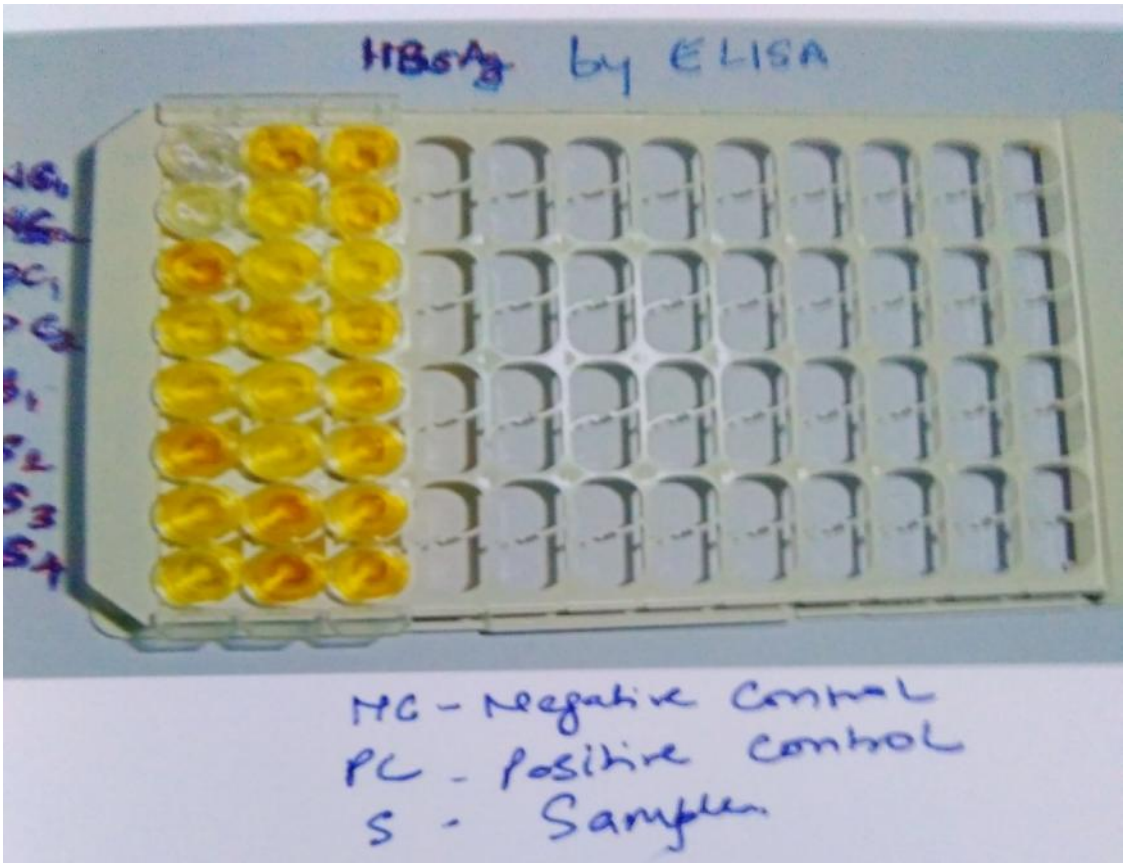
For HBV DNA

2 ml of blood in purple capped (EDTA) vials was sent to Thyrocare Mumbai. Real time Polymerase Chain Reaction based on dual labeled hybridization probe targeting the pre-core and core region was done. DNA quantitation was done on patient plasma (500 µl) using COBAS TAQ man HBV test with high pure extraction, as per manufacturer`s protocol. Results were expressed in IU/ml.

Taqman principle: Forward and reverse primers hybridize during PCR to form a specific sequence product. Taqman probe, an oligonucleotide labeled with a reporter dye is present in the same reaction mixture. It hybridizes to target sequence within the PCR product. A Taqman polymerase that has exonuclease activity cleaves the probe. Upon cleavage, the reporter dye and quencher dye are separated. This results in increase in fluorescence for the reporter. The increase in fluorescence is directly proportional to target amplification.

HBsAg ELISA Kit

ELISA READER



HBsAg by ELISA

RESULTS

Study population includes 30 chronic carriers of Hepatitis B Virus who have tested positive for serum HBsAg twice at an interval of 6 months.

AGE & SEX DISTRIBUTION

The Age wise distribution shows that majority of patients belong to age group 20-30 years (46.7%). Range of age is 20-75 years. Mean age is 34.03.

Males are 40% (12) and females are 60% (18).

RISK FACTOR ANALYSIS

The factors that are taken into consideration are blood transfusion, surgery, other invasive procedures, family history, frequent injections, tattooing and promiscuous behavior (life-style).

Most frequent risk factor identified is therapeutic injections (36.7%) followed by family history (20%).

SEROLOGICAL PROFILE

All patients positive for HBsAg have been included in the present study.

HBeAg positivity was found in 23.3% (7) patients. Most of the HBeAg positive patients belonged to age-group less than 40 years.

No significant association was found between HBeAg positivity and gender.

HBV VIRAL LOAD PROFILE

63.3% (19) people had viral DNA below detectable levels.

13.3% (4) had < 200 IU/ml.

10% (3) had between 201-2000 IU/ml.

6.7% (2) between 2001-20,000 IU/ml.

6.7% (2)> 20,000 IU/ml super carriers.

ASSOCIATION OF HBV VIRAL LOAD AND HBeAg

Out of 7 HBeAg positive people,

43% (3) people had high load of viral DNA (>20,000IU/ml or 10^5 copies/ml).

14% (1) person had between 2000-20,000IU/ml or (10^4-10^5 copies/ml).

14% (1) person had between 201-2000 IU/ml (10^3-10^4 copies/ml).

29 %(2) less than 200IU/ml (<10^3 copies/ml).

Significant association has been found with p value 0.001 between viral load and HBeAg.

CATEGORIES OF CHRONIC HBV CARRIERS ACCORDING TO THEIR SEROLOGIICAL AND VIROLOGICAL PROFILE

HBeAg negative patients were characterized into chronic inactive group, which is further divided into simple carriers and healthy carriers.

The categories of carriers were as follows

Chronic inactive carrier- 18 (60%).

Healthy carriers- 5 (16.5%).

Simple carriers- 4 (13.3%).

Super carriers – 3 (10%).

Table No. 1: Age wise distribution of patients.

Age	Patients	Percentage
20-30	14	46.7
31-40	6	20.0
41-50	7	23.3
51-60	2	6.7
61-70	1	3.3
71+	1	3.3
Total	30	100.0

Chart No. 1: Age wise distribution of patients.

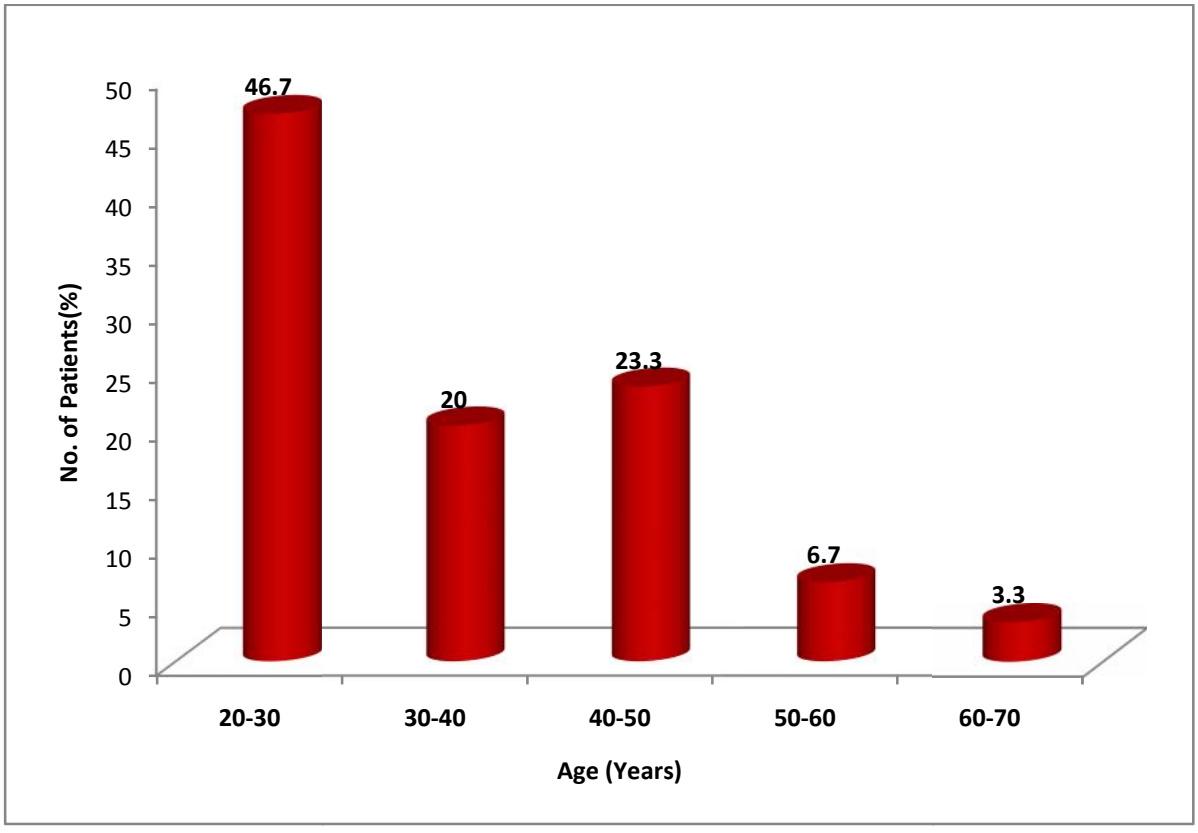


TABLE. No. 2. GENDER WISE DISTRIBUTION OF PATIENTS

Gender	Patients	Percentage
Male	12	40.0
Female	18	60.0
Total	30	100.0

CHART No. 2. GENDER WISE DISTRIBUTION

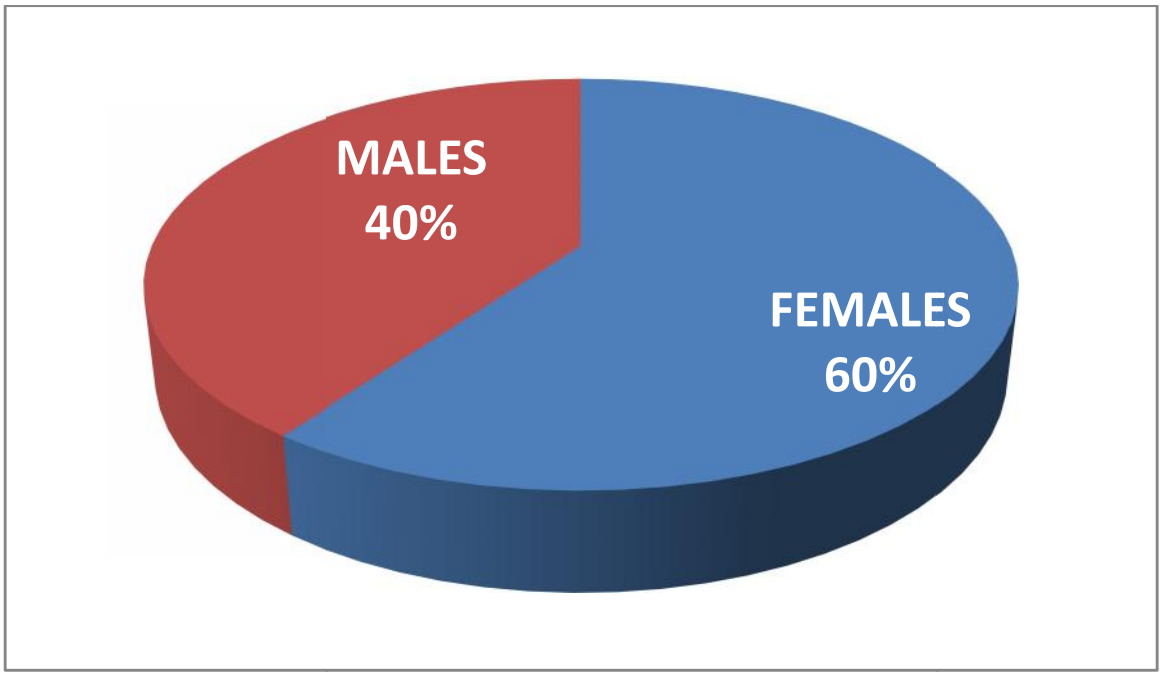


TABLE No. 3. ANALYSIS OF RISK FACTORS

Risk Factors	**Patients**	**Percentage**
Injections	11	36.7%
Family History	6	20%
Blood transfusion	1	3.3%
Life-Style	1	3.3%
Unknown	11	36.7%

CHART No. 3. ANALYSIS OF RISK FACTORS

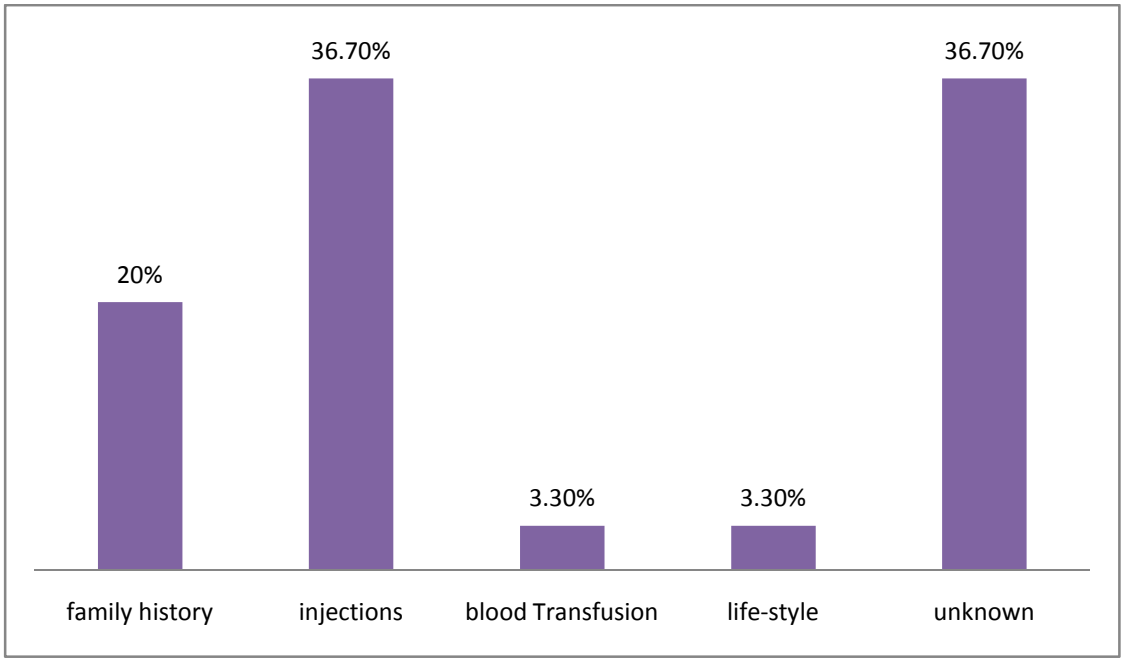


TABLE No. 4. HBeAg STATUS OF PATIENTS

HBeAg status	Patients	Percentage
Positive	7	23.3
Negative	23	76.7
Total	30	100.0

CHART No. 4. HBeAg STATUS OF PATIENTS

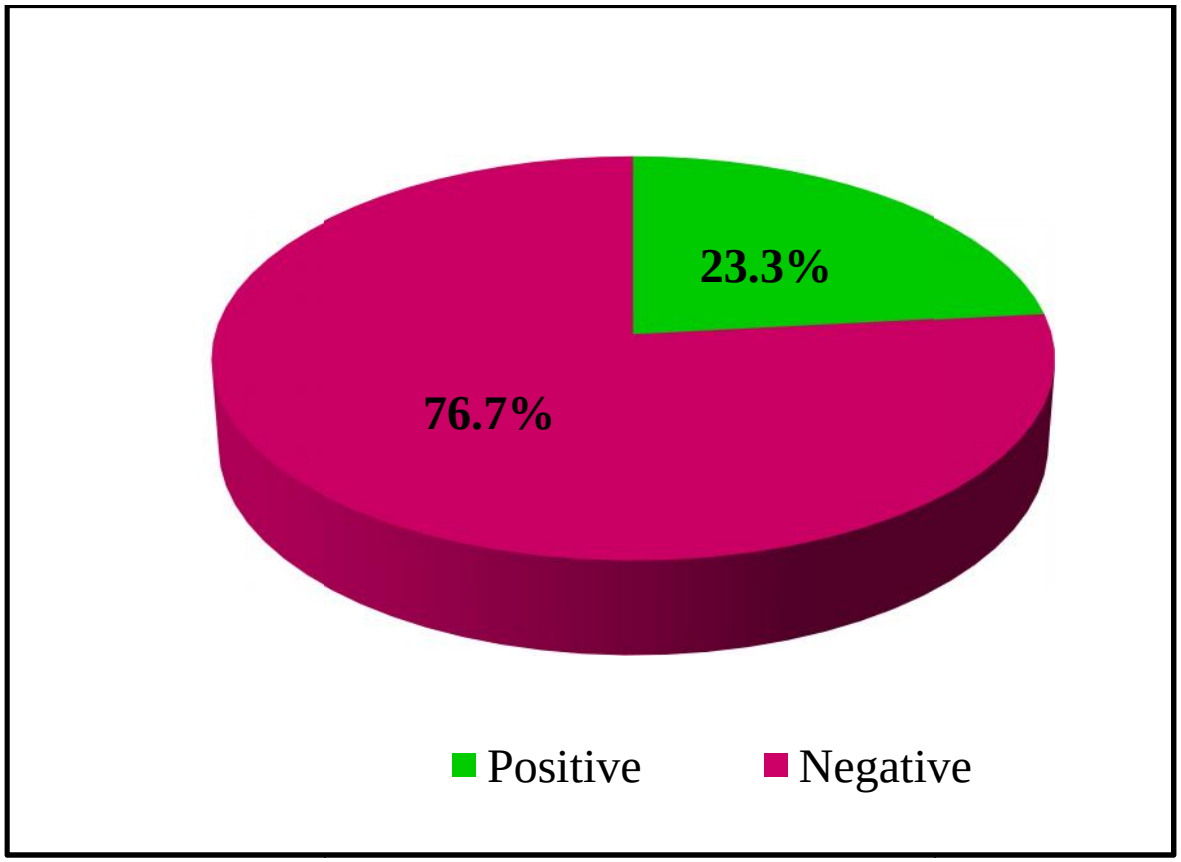


Table No. 5: Viral DNA LOAD OF PATIENTS

Viral DNA	Patients	Percentage
50<200	3	10
201-2000	3	10
2001-20000	2	6.7
>20000	3	10
Below detectable	19	63.3
Total	30	100.0

CHART No. 5. Viral DNA LOAD OF PATIENTS

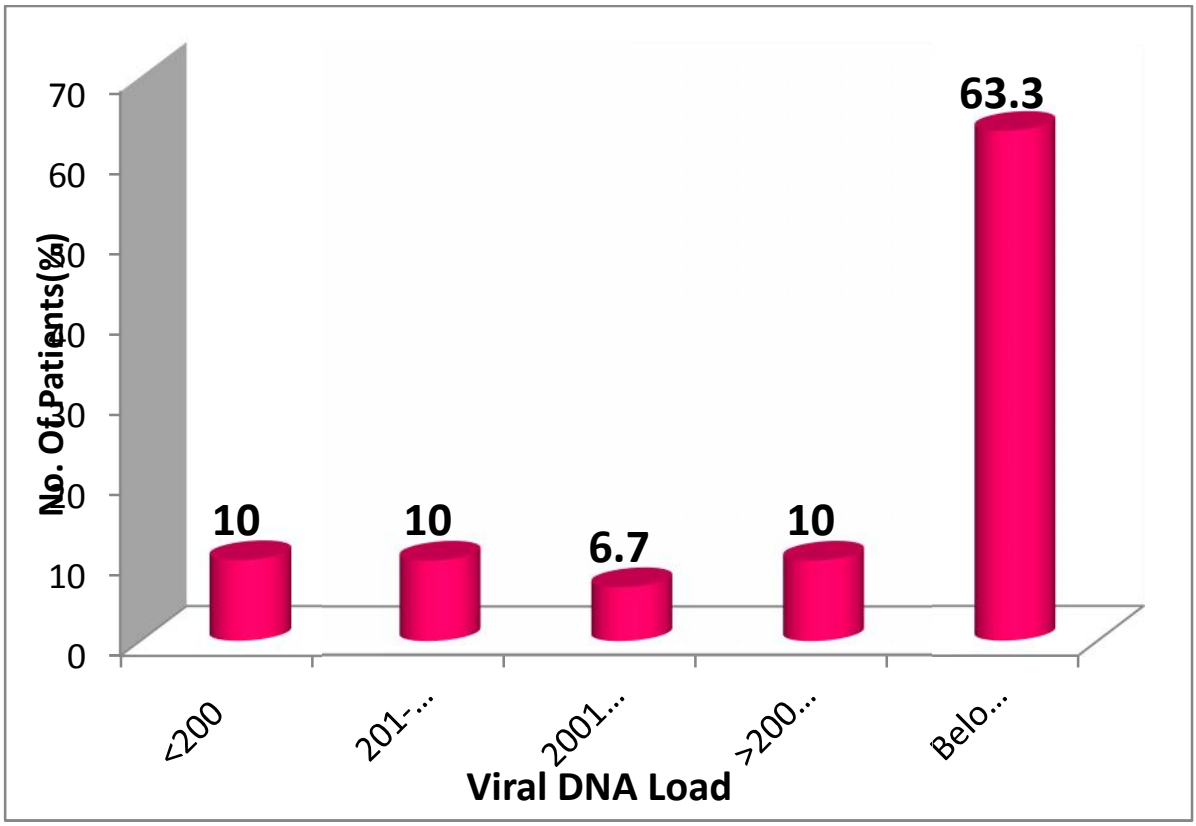


Table No. 6. Distribution of HBeAg Among various Age groups

Age	*** HBeAg status**		**Total**	**chi square test p value**
	Negative(%)	Positive(%)		
20-30	8(57.1)	6(42.9)	14	P=0.196
31-40	6(100)	0	6	
41-50	6(85.7)	1(14.3)	7	
51-60	2(100)	0	2	
60+	1(100)	0	1	
Total	23(76.7)	7(23.3)	30	

Table No. 7.

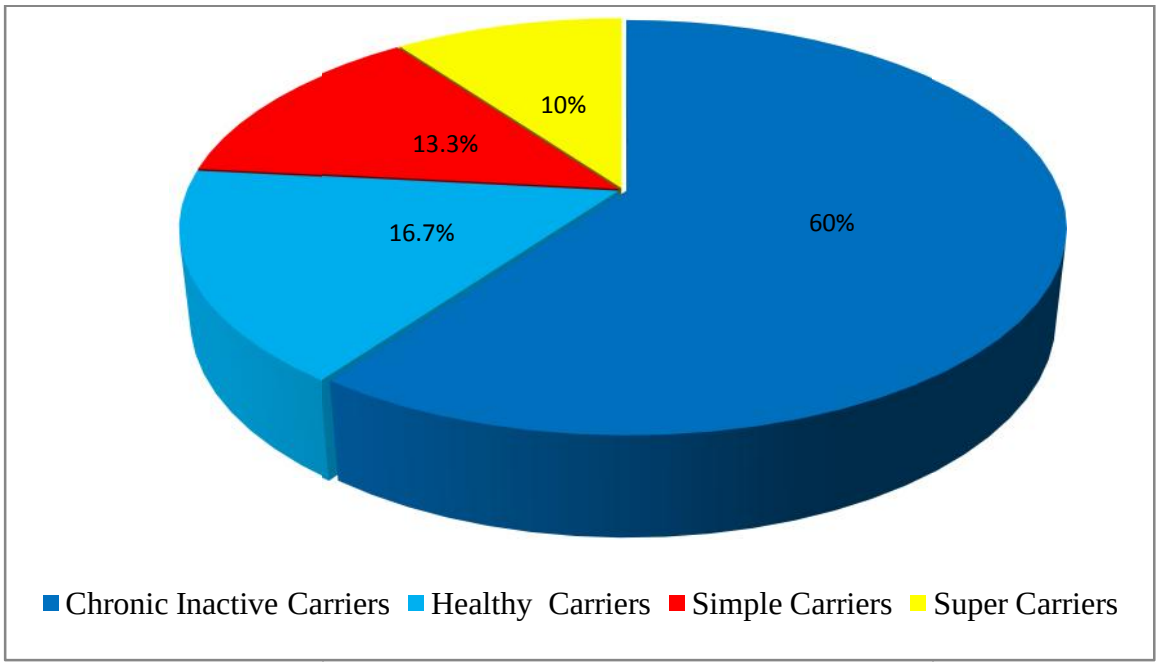
Association between HBeAg positivity and age groups

Age	*** HBeAg status**		**Total**	**Chi square test**
	Negative	Positive		
≤40	16(69.6%)	7(30.4%)	23(100.0%)	P=0.096 NS
>40	7(100%)	0	7(100%)	
Total	23(76.7%)	7(23.3%)	30(100%)	

Table No. 8.Distribution of patients asTypes of carriers

Carriers	No.of Patients	Percentage
Chronic Inactive Carriers	18	60
Healthy Carriers	5	16.7
Simple Carriers	4	13.3
Super Carriers	3	10
Total	30	100.0

Chart No. 6.Types of carriers



DISCUSSION

About two billion people across the world are infected with hepatitis B and there are about 350 million chronic carriers. In India, the carrier rate has been found to be around 4-7% with an estimated 45 million individuals infected with the virus.

The hepatitis B virus resides in the human carrier pool;the infection may lead to a wide spectrum of liver diseases which range from acute to chronic hepatitis, cirrhosis and hepatocellular carcinoma.[3]

Molecular advances in the diagnosis of HBV infection: Nowadays, real time PCR for HBV DNA is a sensitive test and has an excellent level of performance. It detects, theoretically, minimum of 1 DNA molecule/reaction mix up to 10^7 or more.

In 1991, WHO introduced 1IU= 5 molecules of DNA (variable according to the method of assay). National institute of health (NIH), USA, in a workshop on hepatitis B, recommended the use of a new term, inactive HBsAg carrier state`` in place of healthy asymptomatic carriers. It was also proposed that this class of HBV carriers is better defined by an arbitrary HBV DNA level below 10^5copies/ml of the serum and the patients having levels more than this value should undergo antiviral treatment, irrespective of the HBeAg status, contrary to previous concept.

One of the most common ironies in case of chronic hepatitis B patient is the differentiation between HBeAg negative chronic hepatitis cases from inactive carriers as they have a common serological profile.

The determination of HBV in plasma and serum is superior to HBeAg testing as it reflects the replicative activity of HBV more accurately.

The level of HBV replication is the most important risk factor for the eventual development of cirrhosis, hepatocellular carcinoma in both HBeAg reactive as well as HBeAg non-reactive patients.

The three major liver societies, the American Association for the study of Liver diseases, the European Association for the study of the Liver and the Asia-Pacific association for the study of the Liver, have all issued guidelines for the management of CHB that specify certain HBV DNA thresholds to determine which patients are potential candidates for anti-viral treatment.

In view of these facts, this cross- sectional study has been planned to assess the serological markers, HBsAg and HBeAg in the chronic carriers of hepatitis B, and also to compare them with the viral load as determined by the Polymerase chain reaction assay.

In the present study, 30 chronic carriers of hepatitis B were enrolled. The study includes asymptomatic subjects who were detected during; routine screening of blood donors, family contacts of HBV positive individuals, ante-natal checkup and pre-operative evaluation of patients.

The study group was evaluated by detailed history taking and the details of laboratory tests were recorded according to the proforma.

The duration of the study period was from January 2016 to June 2017.

Quantitative estimation of HBV DNA was done by PCR assay and HBsAg , HBeAg were obtained from the laboratory.

The results obtained were subjected to appropriate statistical analysis.

PROFILE OF THE STUDY GROUP

AGE & GENDER

The age of the subjects in the present study ranged from 20-75 years with a mean age of 34.03 years. Ashis Mukhopadhya[69]*et al* found mean age to be 33.1 years. Most of the people belong to 20-30 years age group (46.7%).

86% of the patients who were HBeAg positive fall in the age group of 20-40 years.

In our study of 30 patients, males were 12 (40%) and females were 18 (60%).

The correlation between gender and HBeAg positivity was not significant.

HBeAg positive patients tend to be younger when compared to HBeAg negative patients. Similar findings were reported in other studies conducted by El-Hazmi *et al*[63], Dixit *et al*[64].

Joseph Forbi*et al.*[65]studied the prevalence of HBeAg in chronic HBV carriers in North Central Nigeria and concluded that HBeAg was more prevalent in males 81.8% and less in females 18.2%. Prevalence of HBeAg appears to be higher in males than in females ($p<0.05$). Age groups 0-10 years and 11-20 years had a higher prevalence of HBeAg which decreased with increase in age.

D W Taura*et al.*[66] studied HBeAg antigenemia and development of hepatocellular diseases in Kano Nigeria and found that the rate of HBeAg was higher in males than in females($p<0.05$) and the most susceptible age group for HBeAg infection was ≤ 20 years. It could be because of immature immune response of the children. In endemic areas, mother to child transmission accounts for almost 50% of chronic cases and this also supports this age distribution of HBeAg.

Shamima Akthar*et al.*[67] found that there was male preponderance of HBeAg prevalence, ratio being M:F::4.08:1.

Raihan and Majid in separate studies in Bangladesh observed male preponderance with ratio being 4.8:11 and 3.76:1 respectively. [67]

A study by Mohammed Taghi Shakeri[68]*et al* observed males 1.6% and females 0.7% ($p=0.029$). However, they found lowest prevalence of HBeAg in 35-40 years age group.

RISK FACTORS

The detailed history taken revealed certain risk factors in the study group viz ; blood transfusions, surgery and other procedures, therapeutic injections and sexual promiscuity. Among the various risk factors, therapeutic injections were found to be the most common (36.7%). Similar findings were observed by the study conducted by Shanmugam*et al.*[70] This could probably be due to the use of unsterile methods for administering therapeutic injections.

Singh *et al.*[23] in their study reported unsterile methods and syringes as an important risk factor in the development of an outbreak of viral hepatitis in Andhra Pradesh and also in some rural areas in Gujarat and Haryana. These studies firmly establish the role of unsterile methods as the mode of transmission of Hepatitis B, particularly in the rural population.

The other risk factors like family history found in 20%, promiscuous life-style in 10%, blood transfusion in 3.3% of cases.

Our findings are consistent with other study conducted by Shanmugan*et al.* [70] at Chennai.

Hatami *et al.*[71], Iran, in their study on "Intrafamilial transmission of hepatitis B virus infection" observed that the prevalence of HBsAg positivity was four times more common among family members than the general population.

The occurrence of HBV positivity was more in mothers of the cases than their spouses. Hence it substantiates the fact that perinatal transmission is more important in the spread of infection when compared to sexual transmission in the developing nations.

Our study had 12 women diagnosed to be HBsAg positive during pre-pregency early pregnancy or in previous pregnancies and were also HBsAg positive after delivery. They were evaluated for HBeAg status and viral DNA in their post-natal period. This specific group had 7 women (58.3%) were HBeAg negative and 5 (41.7%) were HBeAg positive.

All of the HBeAg positive women had detectable load of viral DNA.

The specific group of health care providers, I.V drug abusers were not found to be risk factors in our study. This may be due to a small sample size of our study.

SEROLOGICAL PROFILE

The present study consists of chronic carriers of Hepatitis B virus (HBsAg positive for more than six months duration).

The serological marker HBeAg status was obtained in all these patients and they were grouped into HBeAg positive and HBeAg negative groups.

In this study, HBeAg positive were 23.3% patients and remaining 76.7% were HBeAg negative.

Our findings are in consonance with those observed in the study by Shammugam*et al*. [70] Their study reported replicative carriers to be 23.4%.

Quantitative Detection of HBV DNA

This was done in all 30 patients in their plasma by Polymerase Chain Reaction assay.

In the present study, the analysis of HBV DNA load showed that most (63.3%) were negative for viral DNA in their plasma; it was detected in varying amounts in the remaining 36.7%.

In the studies conducted by Hasan N K*et al*[72] and Rabbi F J[73] *et al*, the results showed HBV DNA positivity in 44.8% and 40.2% respectively. Our findings are also in accordance with these studies.

However, the results of the studies by Behnava B*et al*[74] and El Hazmi *et al*[63]showed increased number of positive cases which is in discordance with our results.

In the HBV DNA positive cases the values ranged in between 50 IU/ml to 64,291,972.91 IU/ml.

SEROLOGICAL & VIROLOGICAL PROFILE

The levels of HBV DNA were compared with the serological profile i e HBeAg positive and HBeAg negative in our study.

The results showed that 100 % HBeAg positive were also HBV DNA positive ranging from 66 IU /ml to > 20000 IU/ml and most of them had high viremic levels > 20000 IU /ml.

This is in accordance with the studies by Widita H*et al*[75] done in Indonesia and by Yalein K*et al*[76] done in Turkey.

83% of HBeAg negative patients had no detectable DNA.

17% had detectable DNA 50 IU/ml – 5544 IU/ml with low viremic range.

There is association between HBeAg and viral DNA(p=0.001).

The results of the various studies conducted by Rabbi[73]*et al*, Hasan*et al*[72] and Shammugham *et al*[70], the HBeAg negative group had DNA positive status in 31.5%, 7.6% and 7% respectively.

Our results are in consonance with the study.

X Liu*et al*[51] HBsAg &HBV DNA showed positive correlation in HBeAg positive group ($p<0.001$) and no correlation was found in HBeAg negative group ($p=0.825$). HBV DNA expression correlated with HBeAg ($p<0.001$) and pre-S1 antigen ($p<0.001$)

HBeAg and HBV DNA are not associated ($k =0.29$) and in this study, 40.04% of HBeAg negative patients showed HBV DNA replication. This could be related to mutations in pre-C region.

Thus, HBeAg is useful in Hepatitis B infection for diagnosis and treatment but it cannot replace HBV DNA when we consider HBeAg negative CHB patients. HBeAg detection helps in only some types of CHB cases and it can also be used as a complementary test.

R M Mukherjee[42]*et al* concluded that S.HBsAg concentration was independent of S HBV DNA levels and the status of immune cells. However, HBV suppressed the total T cell population; specifically the cytotoxic T cells population in circulation and this was more significant in patients with higher viral load.

Shamima Akhter[67]*et al* observed a positive correlation among HBeAg and HBV DNA in chronic carriers, however some discordance was observed. Assessment of HBV DNA is also advocated in chronic carriers.

X Liu[51]*et al* found that correlation coefficient between HBsAg and HBV DNA increased with increase in HBV DNA levels and were significant at high values of DNA ($\log_{10}> 7$) $p< 0.001$.

Azita Ganji[77] *et al* concluded that HBeAg negative patients had lower levels of HBV DNA.

Single point HBsAg and HBV DNA quantification gives the most accurate identification of chronic carriers.

Maimuna E Mendy[78] *et al* concluded that HBV DNA was a more reliable indicator of the presence of HBV than HBeAg antigen and was detected in 77% of HBeAg negative and all of the HBeAg positive carriers.

Categories of carriers on the basis of their viral DNA can be grouped as chronic Inactive carriers -18 (60%), Healthy carriers -5 (16.5%), Simple carriers-4 (13.3%) and Super carriers-3 (10%).

HBV DNA quantification by qPCR is a reliable, accurate & reproducible test and can be used to diagnose, monitor the efficacy of therapy and understand the natural history of the disease.

CONCLUSION

Our country has a huge pool of hepatitis B patients and asymptomatic carriers are the main reservoir which silently transmits the infection in the community. This study was aimed at detecting the presence or absence of HBeAg among these people and co-relating the information with their viral load.

The purpose was to highlight that, the carrier state, though seems benign, is prone to progression of disease and complications and hence requires diagnosis, monitoring, counseling regarding blood and organ donation and prompt treatment when required.

Our study revealed that that majority of the patients were chronic inactive carriers who were healthy and may have total HBsAg clearance later in life. The risk of transmission was also very low as viral load was below detectable levels. However, it is not wise to look upon them for blood donation or as solid organ transplant donors.

HBeAg positive status predicted infectivity and also a risk of disease progression to chronic hepatitis B followed by the ominous cirrhosis and hepatocellular carcinoma.

The role of HBV DNA PCR cannot be overemphasized.

It is also helpful in the diagnosis of HBeAg negative chronic hepatitis B patients. Their serological profile is same as inactive carriers and this test solves the clinical dilemma.

Serial measurement of HBV DNA levels and routine evaluation of these patients are advocated. Pregnant women have to be actively monitored and lactating mothers along with their new-born babies are to be targeted for active-passive immunization to decrease the prevalence of disease in community.

SUMMARY

- The study population consists of 30 asymptomatic chronic carriers, age ranging from 20years to 75 years with majority of the people coming from age-group 20 years -30 years.
- Majority of the HBeAg positive carriers belonged to the age group less than 40 years. Replicative carriers are young and seroconversion occurs at a later age.
- Males were 40% and females constituted 60% of the study group.
- The most common risk factor in the group was frequent therapeutic injections 36.7%, highlighting the need for safe injection practices at grass root levels.
- Family history was next most important risk factor 20% which proves that horizontal transmission among close family contacts was a major route of transmission in our country.
- The number of HBeAg positive patients was 23.3% with no significant association with gender.
- HBV DNA was positive in all 23.3% HBeAg patients with 3 patients showing very high levels of DNA.
- All replicative carriers(HBeAg +) are viremic.
- The categories of carriers are as follows
- Chronic inactive carrier-18(60%)
- Healthy carriers-5(16.5%)
- Simple carriers-4(13.3%)
- Super carriers – 3 (10%)

- Ante- natal cases deserve a special mention as the immune status returns back to normal in post-partum period and flares of hepatitis are likely.
- The newborn of a carrier mother is to be immunized actively as well as passively. After this, there is no contraindication to breast-feeding. This immunization is one of the most effective methods of decreasing the carrier pool of the virus.

LIMITATIONS OF THE STUDY

- Single centric study with Small Sample size.
- The markers which reveal the behavior of virus in the host i.e. Presence of HBsAg, HBeAg and HBV DNA have been studied. The whole range of markers which quantitate the host response to the virus have not been included. The clinical parameters like ALT, AST have not been taken into account.
- A vertical multi centric study with a large sample size, active inputs by clinicians is recommended in which parameters that quantitate the same will be studied.

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ANNEXURES

ETHICAL CLERANCE CERTIFICATE

B.L.D.E.UNIVERSITY'S
SHRI.B.M.PATIL MEDICAL COLLEGE, BIJAPUR – 586103
INSTITUTIONAL ETHICAL COMMITTEE

No/58/2015
20/11/15

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this college met on 17-11-2015 *at* 03 pm *scrutinize the Synopsis of Postgraduate Students of this college from Ethical Clearance point of view. After scrutiny the following original/corrected and revised version synopsis of the Thesis has accorded Ethical Clearance.*

Title "Serological Markers HBsAg and HBeAg in chronic hepatitis B carriers and their correlation with viral load by polymerase chain reaction assay"

Name of P.G. Student : Dr, Rasika Borgaonkar
Dept of Microbiology

Name of Guide/Co-investigator : Dr P. R. Sholapure
Prof & HOD.

DR.TEJASWINI VALLABHA
CHAIRMAN

CHAIRMAN
Institutional Ethical Committee
BLDEU's Shri B.M. Patil
Medical College,BIJAPUR-586103.

Following documents were placed before E.C. for Scrutinization
1)Copy of Synopsis/Research Project
2)Copy of informed consent form.
3)Any other relevant documents.

INFORMED CONSENT FORM

TITLE OF TOPIC	**:**	**SEROLOGICAL MARKERS HBsAg& HBeAg IN CHRONIC HEPATITIS BCARRIERS AND THEIR CORRELATION WITH VIRAL LOAD BY POLYMERASE CHAIN REACTION ASSAY**
PRINCIPAL INVESTIGATOR	**:**	**Dr. RASIKA BORGAONKAR**
PG GUIDE NAME	**:**	**DR.PRAVEEN SHAHAPUR**

PROCEDURE:

I am aware that in addition to routine care received & I will be asked series of questions by the investigator. I understand that my blood sample will be subjected to various investigations needed for research purpose.

RISK AND DISCOMFORTS:

I understand that I may experience some discomfort during collection of Blood sample. This is mainly the result of my condition & the procedures of the study are not expected to exaggerate these feelings which are associated with the usual course of the treatment.

BENEFITS:

I understand that my participation in the study as one of the study subject will help theresearcher .. Study will not have direct benefits to me other than the potential benefits of the study for choosing appropriate treatment.

CONFIDENTIALITY:

I understand that the medical information produced by this study will become a part of hospital records & will be subject to confidentiality. Information of sensitive personal nature will not be part of the medical record, but will be stored in the investigators research file & identified only by code number, the code key connecting name to numbers will be kept in separate secure location.

If the data are used for publication in the medical literature or for teaching purpose, no name will be used.

I understand that the relevant designated authorities are permitted to have access to my medical record & to the data produced by the study for audit purpose, however they are required to maintain confidentiality.

REQUEST FOR MORE INFORMATION:

I understand that I may ask more questions about the study at any time. Dr.RasikaBorgaonkar at the department of Microbiology is available to answer my questions or concerns. I understand that I will be informed of any significant new findings discovered during the course of the study, which might influence my continued

participation. A copy of this consent form will be given to me to keep for careful reading.

REFUSAL OR WITHDRAWAL OF PARTICIPATION:

I understand that my participation is voluntary and that I may refuse to participate or may withdraw consent and discontinue participation in the study at any time without prejudice. I also understand that Dr. RasikaBorgaonkar may terminate my participation in the study at any time after she has explained the reasons for doing so.

INJURY STATEMENT

I understand that in the unlikely event of injury to me resulting directly from my participation in this study, if such injury were reported promptly, the appropriate treatment would be available to me. But no further compensation would be provided by the hospital. I understand that by my agreements to participate in this study I am not waiving any of my legal rights.

I have explained to Mr/Ms_____ the purpose of the research, procedures required & the possible risks to the best of my ability.

_____

Dr. RasikaBorgaonkar Date:

(Investigator)

STUDY SUBJECT CONSENT STATEMENT:

I confirm that Dr. Rasika Borgaonkar has explained to me the purpose of research, the study procedure that I can undergo & the possible discomfort as well as benefits that I may experience in my own language. I agree with full conscious to give consent to participate as subject in the research project.

_____ Date:

(participant signature)

PROFORMA

Name of Patient :

Age /Gender :

O.P.D /IPD No :

Department :

Past History :

H/O Jaundice, Blood Transfusion, Frequent Injections, Surgery, Dialysis, Hepatitis B vaccination, Chronic Drug Intake.

Personal History:

Chronic Smoking, Drug Intake, Pre/Extra-Marital Sex, Alcohol Intake, Tattooing.

Family History: First Degree Relatives positive for HBsAg, Diagnosed or Died of HCC or chronic liver disease.

Clinical diagnosis

HBSAg status 1st detected.

HBsAg HBeAg Viral DNA load

Date

APPENDIX

ELISA: Other Materials Required:

- Manual pipette
- Pipette tips
- Incubator
- Absorbent sheets
- ELISA micro plate Reader
- Disinfectant
- Reagent Grade Water
- Disposable gloves
- Timer
- Biohazard waste container
- Serological pipettes
- Sterile dry tubes for conjugate activation

Kit Components

- Coated microwells
- Positive and Negative controls
- Conjugate along with conjugate activator
- Substrate
- Wash buffer
- Stop solution

Micro well holder

KEY TO MASTER-CHART

M	-	Male
F	-	Female
BT	-	Blood Transfusion
PCR	-	Polymerase Chain Reaction
HBV DNA	-	Hepatitis B Virus Deoxyribonucleic Acid
IU	-	International Units
1 IU	-	5.82 copies/ml
HBeAg	-	Hepatitis B e Antigen
Y	-	Yes
P	-	Positive
N	-	Negative
TND	-	Target Not Detected less than 20 IU/ml

MASTER CHARTS

S. no	Name	Age	Sex	I.P/OPD no	Family History	Injections	post-natal	Blood transfusion	Life-Style	Date of detection	Present HBsAg status	HBeAg status	Viral DNA IU/ml
1	Savitri bai	35 yrs	F	22757	N	Y	Y	N	N	13/5/2012	POSITIVE	Negative	TND (below detectable)
2	Hajmahussini	31 yrs	M	248691	Y	N	NA	N	N	6/9/2015	POSITIVE	Negative	below detectable
3	Chandu Lamani	35 yrs	M	248696	N	Y	NA	N	N	18/6/2016	POSITIVE	Negative	below detectable
4	Laxmi melli	43 yrs	F	232730	N	Y	NA	N	N	1999	POSITIVE	Negative	576 IU/ml
5	Savitri bai	41 yrs	F	232757	N	N	NA	N	N	12/2/2010	POSITIVE	Positive	745 IU/ml
6	M.K. Desai	38 yrs	M	232758	N	N	NA	N	Y	2012	POSITIVE	Negative	280 IU/ml
7	Jayabheem Zalaki	27 yrs	M	232761	Y	N	NA	N	N	12/1/2010	POSITIVE	positive	2459 IU/ml
8	Boramma Hiremath	58 yrs	F	248702	N	Y	NA	N	N	26/1/12	POSITIVE	Negative	below detectable
9	Asma Kalebag	21 yrs	F	232762	N	N	Y	N	N	2/8/2010	POSITIVE	Negative	below detectable
10	Savitri Patil	48 yrs	F	232766	N	N	NA	N	N	19/7/2007	POSITIVE	Negative	below detectable
11	Vaibhav	21 yrs	M	42850	N	N	NA	N	Y	30/12/2016	POSITIVE	Negative	below detectable
12	Kamla bai	40 yrs	M	39546	N	Y	NA	N	N	3/12/2016	POSITIVE	Negative	below detectable
13	Bhimraya	58 yrs	M	8800	N	N	NA	N	Y	19/3/2016	POSITIVE	Negative	below detectable
14	Sadanand	30 yrs	M	90031	N	Y	NA	N	N	10/3/2016	POSITIVE	Negative	below detectable
15	Indra bai	28 yrs	F	23290	N	Y	Y	N	N	12/1/2015	POSITIVE	Positive	66IU/ml
16	Shreedevi	21 yrs	F	22902	N	Y	Y	N	N	11/9/2016	POSITIVE	Negative	50IU/ml
17	Kulsumbee Gachamahal	42 yrs	F	248708	Y	N	NA	N	N	8/1/2013	POSITIVE	Negative	5544 IU/ml
18	Santosh	25 yrs	M	22211	N	N	NA	N	N	8/1/2015	POSITIVE	Negative	below detectable
19	K.B Patil	75 yrs	M	11384	Y	N	NA	N	N	10/1/2010	POSITIVE	Negative	below detectable

20	Kaveri	20 yrs	F	24460	N	Y	Y	N	N	10/10/2012	POSITIVE	Positive	64,291,972.9 IU/ ml
21	Neelganga Dattu Kadagoal	22 yrs	F	24461	N	N	Y	Y	N	7/2/2005	POSITIVE	Positive	5831742 IU/ml
22	Latha Hosamani	20 yrs	F	24790	N	N	Y	N	N	25/12/16	POSITIVE	Positive	126693 IU/ml
23	Chandrakala	21 yrs	F	225495	N	N	Y	N	N	12/3/2016	POSITIVE	Negative	below detectable
24	Shankarawwa	45 Y	F	21387	Y	N	NA	N	N	23/12/16	POSITIVE	Negative	below detectable
25	Basalingamma	26 Y	F	20849	N	Y	Y	N	N	12/12/2016	POSITIVE	Negative	below detectable
26	Husanbee	28 yrs	F	25885	Y	N	Y	N	N	2008	POSITIVE	Negative	below detectable
27	Sidram Asangi	35 yrs	M	26986	N	Y	NA	N	N	12/9/2015	POSITIVE	Negative	below detectable
28	Ramanna	40 yrs	M	27159	N	N	NA	N	N	22/12/14	POSITIVE	Negative	below detectable
29	Bhagyashree	20 yrs	F	26806	N	N	Y	N	N	14/3/16	POSITIVE	Positive	200 IU/ml
30	Amita	28 yrs	F	25930	N	N	Y	N	N	14/12/15	POSITIVE	Negative	below detectable